Hepatic Encephalopathy: The role of Inflammation, Ammonia and Aquaporin Expression in the Pathogenesis of Cerebral Oedema

By

Dr Gavin A.K Wright

Thesis submitted for the degree of *'Doctor of Philosophy'* (PhD)

Liver failure group
The Institute of Hepatology, University College London

September 2009

‘I, Dr Gavin Wright confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.'

Signature:

Date:

“Science is a way of thinking much more than it is a body of knowledge”

Dr. Carl Sagan

Abbreviations

AA	Arachidonic acid
Ach	Acetylcholine
α-KG	Alpha-ketoglutarate
AD	Alzheimer's disease
ADMA	Asymmetric dimethyl-L-arginine
ADP	Adenosine di-phosphate
ALF	Acute liver failure
ALT	Alanine aminotransferases
ACLF	Acute-on-chronic liver failure
AQP	Aquaporin
Asp	Aspartate
AST	Aspartate aminotransferases
ATP	Adenosine tri-phosphate
A-V	Arterial-venous
BCAAs	Branched-chain amino acids
BDL	Bile duct ligation
CBF	Cerebral blood flow
Cho	Choline-containing compounds
CLD	Chronic liver disease
CLP	Caecal ligation and puncture
COX	Cyclooxygenase
CPP	Cerebral perfusion pressure
CPT	Carbamoyl-phosphate synthetase

Cr	Creatine
CRP	C-reactive protein
CYP	Cytochrome
DCT	Distal collecting tubules
DDAH	Dimethylarginine dimethylaminohydrolase
DST	Digit symbol test
EAAC	Excitatory amino acid carrier
EAAT	Excitatory amino acid transporter
EEG	Electroencephalogram
EET	Epoxyeicosatrienoic acid
EM	Electron-microscopy
eNOS	Endothelial NO synthase
FBI	Focal brain ischaemic
GABA	Gamma aminobutyric acid
GDH	Glutamate dehydrogenase
GI	Gastrointestinal
GALN	Galactosamine
GLN	Glutamine
GLT	Glutamate transporter
GLU	Glutamate
cGMP	Cyclic guanosine monophosphate
GS	Glutamine synthetase
HAL	Hepatic artery ligation
HCO_3^-	Bicarbonate
HD	Hyperammonemic diet
HE	Hepatic encephalopathy

HETE	Hydroxy eicosatetraenoic acid
HPLC	High performance liquid chromatography
HRS	Hepatorenal syndrome
ICP	Intracranial pressure
IFN-γ	Interferon-gamma
IL	Interleukin
IM	Intramuscular
iNOS	Inducible NO synthase
IP	Intraperitoneal
IV	Intravenous
LFTs	Liver function tests
Ln	Lanthanum nitrate (ionic tracer)
LOLA	L-ornithine, L-aspartate
LPS	Lipopolysaccharide (bacterial)
MAP	Mean arterial pressure
MAPK	Mitogen-activated protein kinase
MARS	Molecular adsorbents recirculating system
MDA	Malondialdehyde
mRNA	Messenger ribonucleic acid
MHE	Minimal hepatic encephalopathy
MRI	Magnetic resonance imaging
MTAL	Medullary thick ascending limb
MV	Microvessel
Myo-Ins	Myo-inositol
NA	Noradrenaline
NAA	N-acetyl-aspartate

NCT	Number connection test
NH_3	Ammonia
NH_4^+	Ammonium
NMDA	N-methyl-D-aspartate
nNOS	Neuronal NO synthase
NO	Nitric oxide
Nox	Nitrate/nitrite ratio
NS	Non-statistical
NT	Nitrotyrosine
OLT	Orthotopic liver transplantation
OP	L-ornithine, Phenylacetate
PAG	Phosphate-activated glutaminase
PCS	Portacaval shunt
PCT	Proximal collecting tubule
PDH	Pyruvate dehydrogenase
PET	Positron emission tomography
PG	Prostaglandins
PHES	Psychometric HE score
RAAS	Renin-aldosterone-angiotensin system
RBF	Renal blood flow
SBP	Spontaneous bacterial peritonitis
SEM	Standard error of mean
sGC	Soluble guanylate cyclase
SIRS	Systemic inflammatory response syndrome
SMT	Standard medical therapy
SNAT	Sodium-coupled neutral amino acid transporter

SPECT	Single photon emission computed tomography
Ty	Tyrosine
Tau	Taurine
TIPS	Transjugular intrahepatic portosystemic shunts
TLR	Toll-like receptor
TNF-a	Tumour necrosis factor-alpha
VEP	Visual evoked potential
WCC	White cell count

Abstract

Current evidence indicates synergy between hyperammonaemia and inflammation in the brain with liver failure. Utilising animal and laboratory experiments, this thesis explored a number of concise questions focused on progression to brain oedema and coma with hepatic encephalopathy (HE).

STUDY 1: It is unclear whether the background cirrhotic state or hyperammonaemia predisposes to superimposed inflammation. **Question 1**: Does lipopolysaccharide (LPS)-induced systemic inflammation worsen brain oedema in cirrhotic bile duct-ligated rats? and is this associated with blood-brain barrier disruption? altered brain ammonia and/or inflammatory pathways? **Answer 1:** LPS-induced pre-coma/coma and exacerbated cytotoxic oedema, indicating synergy between hyperammonaemia and inflammation associated with brain protein nitrosation.

STUDY 2: New ammonia-lowering therapies targeting multiple organs are necessary. **Question 2**: Does combining L-ornithine and phenylacetate (OP), synergistically improve ammonia reduction? **Answer 2**: L-ornithine and phenylbutyrate synergistically lead to sustained ammonia-lowering and limited oedema; L-ornithine detoxifying ammonia by providing a substrate for glutamine synthesis and phenylacetate renally excreting the glutamine as phenylacetylglutamine.

STUDY 3: Despite apparent synergism, therapies for HE target either hyperammonemia or inflammation, not both. **Question 3**: Can a reduction in ammonia in cirrhotic rats prevent LPS-induced worsening of brain oedema and progression to pre-coma/coma? Does targeting hyperammonaemia and

inflammation together provide therapeutic synergy? **Answer 3***:* Ammonia primes the brain to the deleterious effect of LPS, with the ammonia-lowering effect of OP preventing LPS-induced coma and brain edema.

STUDY 4: Aquaporin-4 (AQP4), a bi-directional astrocyte water channel, is thought to provoke brain oedema in neuropathic disorders. **Question 4***:* Is AQP4 causally involved in the brain oedema associated with models of liver failure? **Answer 4**: AQP4 has no causal role in the brain edema associated with hyperammonemia or inflammation, with or without acute liver dysfunction. However in cirrhosis, AQP4 upregulation, with contemporaneous $p38^{MAPK}$ activation is possibly a compensatory response to inhibit edema formation.

Research questions

CHAPTER 2: Mechanistic study

Role of ammonia & inflammation in hepatic encephalopathy

1. Do systemic changes in ammonia and inflammation act synergistically to effect the progression and complications associated with hepatic encephalopathy (HE) as indicated by the depth of coma or cerebral oedema?
2. With progression of HE, is there an anatomical or functional alteration of the blood brain barrier?
3. Does the bacterial lipopolysaccharide (LPS) challenged bile duct-ligation (BDL) rat model represent clinical of acute-on-chronic liver failure (ACLF)?
4. On a background of hyperammonaemia and systemic inflammation, are the cerebral effects associated with HE due to alterations in brain ammonia and amino acid metabolism, and/or cerebral inflammatory responses?

CHAPTER 3: Interventional study

Targeting ammonia & amino acid metabolism

1. Can the novel therapeutic agent L-ornithine, Phenylacetate (OP), by targeting inter-organ ammonia & amino acid metabolism, ameliorate the hyperammonaemia and development of advanced HE in rats with cirrhosis?
2. What are the interorgan pathophysiological mechanisms involved?
3. Can any ammonia-lowering effect of OP last throughout a prolonged period of administration?

4. By lowering ammonia, can OP lead to a reduction in brain water?

CHAPTER 4: Interventional study

Targeting ammonia & inflammatory pathways

1. Can a reduction in ammonia in rats with cirrhosis prevent LPS induced worsening of brain oedema and progression to pre-coma and coma stages?
2. Does targeting hyperammonaemia and inflammation together provide therapeutic synergy in the treatment HE?

CHAPTER 5: Mechanistic study

Role of Aquaporin 4 in liver failure

1. Are spatial and/or temporal changes in aquaporin-4 (AQP4) involved in the development of pathogenic brain oedema in liver failure?
2. LPS augments cerebral oedema in HE; Does this involve alteration in AQP4 expression as suggested in other disease states?
3. Does any effect of AQP4 expression on such brain oedema involve changes in MAPK expression?

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Chapter 1.1

Hepatic Encephalopathy:

Defining the Syndrome

Introduction

Hepatic encephalopathy (HE) is a neuropsychiatric syndrome that may develop with liver insufficiency. Acute liver failure (ALF) is defined by the presence of HE, which may progress from confusion to coma, and at its most advanced is associated with intracranial hypertension and possible death from brain herniation.(1) In cirrhosis,(2, 3) HE usually occurs insidiously with wide ranging neuropsychiatric disturbances (e.g. psychomotor dysfunction, impaired memory, decreased reaction time, diminished attention, sensory abnormalities and poor concentration). Patients with cirrhosis can deteriorate and may develop cerebral changes indistinct from ALF, termed acute-on-chronic liver failure (ACLF). Ammonia, inflammation and modulation of cerebral blood flow (CBF) autoregulation are central to the development of HE, and with the predisposing factors which trigger acute liver injury, have become important therapeutic targets in the management of HE.

This chapter discusses the nomenclature, staging, diagnosis and investigation of this complex neuropsychiatric syndrome. It also introduces the fundamental factors involved on its pathogenesis, which will be expanded on in later chapters.

Nomenclature

The wide range of neuropsychiatric presentations (especially when subclinical) has made comparative interpretation of studies into HE problematic.(4, 5) This led to the development of consensus terminology by the 'World Congress of Gastroenterology' in 2002 to classify the cause of liver disorder:(6)

- *Type A*: ALF
- *Type B*: portal-systemic bypass without intrinsic hepatocellular disease
- *Type C*: cirrhosis and portal hypertension with portal-systemic shunts

However, this classification should be revised to incorporate the widespread acceptance of the new clinical entity:

- *Acute-on-chronic liver failure (ACLF):* acute liver injury on the background of a chronic liver disease phenotype, which is clinically indistinct from ALF.(7)

However HE can also be described by the varied clinical course of encephalopathy:

- *Acute encephalopathy:* with the time to encephalopathy defining ALF:
    1. *Hyper-acute:* 7 days between onset of jaundice and encephalopathy
    2. *Acute:* 8–28 days
    3. *Sub-acute:* 5–12 weeks
- *Recurrent encephalopathy:* episodic mental alterations in cirrhotic patients even in the absence of a recognised precipitating factor.
- *Persistent encephalopathy:* unresolved neurological deficit, which may persist despite complete reversal of the liver injury (e.g. successful orthoptic liver transplantation) or removal of precipitant.
- *Minimal or subclinical encephalopathy:* despite absence of overt encephalopathy, mild cognitive abnormalities remain, detected only by psychometric or neurophysiologic tests. This is present in 60-80% of patients with established liver disease.

Clinical staging

Although the 'West Haven criteria' (Table 1) is a specific staging classification of altered mental state in liver injury,(8) the 'Glasgow coma scale'(9) as a robust assessment of consciousness in structural and metabolic brain disorders is also an applicable staging system.

DIAGNOSIS

History & Examination: The need for a detailed history and examination are fundamental to the diagnosis of HE given a broad spectrum of differential diagnosis (e.g. vascular, metabolic, intracranial and other neuropsychiatric disorders, (Table 2). To establish a diagnosis of HE it is necessary for there to be a history or clinical evidence of liver disease with or without the presence of a precipitating factor(s), which also include transjugular intrahepatic portosystemic shunts (TIPS) for intractable ascites or variceal bleeding, (Table 3). With early diagnosis there is an increasing reliance on laboratory and imaging modalities to establish a diagnosis of HE. Findings suggestive of the presence of a precipitating factor must not be missed. For example, spontaneous bacterial peritonitis (SBP) usually presents with signs of peritoneal irritation, pain, nausea, vomiting and changes in gastrointestinal motility (diarrhoea or ileus), but these symptoms and signs may be absent and only detected if a diagnostic paracentesis is routinely performed on acutely unwell and hospitalised patients with ascites.

INVESTIGATIONS

Laboratory tests*:* Routine biochemical tests with a full hepatitis screen are necessary to establish the specific cause of liver failure as this may impact on treatment options. The diagnosis of precipitating factors like infection (e.g. cultures) and electrolyte imbalance is critical. Laboratory diagnosis of SBP is confirmed by the presence of ascitic fluid polymorphonuclear count (>250cm^3) or positive ascitic fluid culture, usually monomicrobial (e.g. Escherichia coli or other gram-negative

bacteria). The use of a dipstick test for leukocyte esterase (present in biological fluids) provides a more rapid diagnosis.

Arterial ammonia: There is evidence to suggest that blood ammonia (especially arterial), correlates with severity of HE in ALF (see later). In cirrhosis, any correlation is somewhat unclear, where levels may be normal in stable disease, persistently elevated (e.g. portosystemic shunting) without clinical impact, or in the very high levels where it may or may not be associated with pre-coma or coma.

Imaging: Diagnostic ultrasound *(*U/S) and computer-assisted tomography (CT) are the predominant imaging modalities, to establish the extent and possible cause of liver injury. In regard to HE (in the non-ventilated patient), head CT remains a useful imaging modality, principally to exclude other differential causes of neurobehavioral change. But research into the pathophysiology of HE has required the use of other brain imaging techniques:(10)

- *Magnetic resonance imaging (MRI):* has detected increased manganese deposition in the basal ganglia, with deposition only associated with portal-systemic shunting and chronic liver dysfunction (not ALF).(11)
- *Single photon emission computed tomography (SPECT):* has shown that in cirrhosis changes to the basal ganglia are related to regional alterations of cerebral blood flow (CBF) in the cortex and subcortical regions.
- *Positron emission tomography (PET) studies:* has shown that in cirrhosis, 1) blood brain barrier permeability and cerebral ammonia metabolism is increased and 2) regional ammonia supply and energy utilisation (using the glucose tracer- fluorodesoxyglucose), correlates to regional blood flow.

Electroencephalogram (EEG): This is a useful tool in the diagnosis of HE due to characteristic background slow-wave development of between 4-6Hz, triphasic waves (TW) which consist of a high-voltage positive wave followed by a smaller negative deflection (usually bilaterally synchronous and maximal frontally)(12) and occasionally epiliptiform, Table 1. However, slow-waves and TW's are not pathognomonic as other forms of encephalopathy (e.g. metabolic or toxic) may lead to similar EEG findings. However, in the context of other representative findings may help establish a diagnosis of HE as distinct from a number of otherwise similarly presenting pathologies. Such changes may even persist following liver transplantation.(13) Care with interpreting such findings should be taken as in liver injury patients also have confounding effects of e.g. malnutrition, hyponatraemia, thiamine deficiency (Wernicke's encephalopathy) and other nutritional and electrolyte anomalies can equally cause a number of indistinct EEG findings like diffuse slowing of brain waves.

Evoked potentials: Evoked potentials are objective and quantitative methods of evaluating the function of peripheral and central nervous systems (PNS and CNS) and may assess auditory, somatosensory or visual evoked potentials, with the latter the most common. HE may be associated with prolonged latency of visual evoked potentials (VEPs)(14) (e.g. N2, P2 and N3), which are assigned according to where the positive (P) or negative (N) waves occur (e.g. the second negative wave, N2) and reduced wave amplitudes (P100 and P300). However, correlation with depth or chronicity HE, though more closely associated with sub-clinical presentation, is debated and inferior to psychometric tests,(15-17) limiting its uses to research.

***Neuropsychological tests**:* the use of neuropsychometric battery tests have been limited to research of minimal HE. Although results from most studies have not been easy to compare due to the array of different tests, the common finding in cirrhotic patients is impaired psychomotor speed, visual perception and attention, while verbal ability is unimpaired. Most studies advocate using the 'number connection test' (NCT), 'digit symbol test' (DST) and/or the 'block design test' (BDT) for the diagnosis of minimal HE. However, a standardised test battery, the 'psychometric HE score' (PHES) is frequently used(5) and considered the 'gold standard' in the diagnosis of minimal HE as it covers the spectrum of cognitive changes, has normative age-corrected data and is inexpensive.(6) This uses the line drawing test (LDT), the serial dotting test (SDT), the DST and the NCT (A and B), to examine motor speed and accuracy, visual perception, visuospatial orientation, visual construction, concentration, attention and to a lesser extent memory. Also, a good performance of all tests will also assess attention.

Critical flicker frequency (CCF): CCF measures the ability of the central nervous system to detect flickering light. An intrafoveal light stimulus with defined pulses (wavelength of 650 nm), initially generates red light as a high-frequency pulse (60 Hz) giving the patient the impression of a steady light. This is gradually reduced until the patient has the impression that the steady light had changed to a flicker, registered by pressing a hand-held switch. These changes are directly influenced by cortical activity and thus correlate to possible brain damage. CCF detects a wide range of neuropsychological abnormalities including visual signal processing (e.g. with retinal gliopathy) to cognitive functions like arousal and attention, but cannot detect motor abnormalities. Over the last 40 years has been utilised to establish responses to certain psychotropic drugs and more recently lend itself to the

diagnose a number of neurological disorders such as Multiple sclerosis and Alzheimer's disease. CCF is beneficial as a test for minimal HE compared to other measures as it is not influenced by age, gender, cultural or educational factors. CFF has also proven a useful test for the diagnosis of minimal HE, with Kircheis and colleagues reporting a 55% sensitivity and 100% specificity using a <39 Hz threshold cut-off,(18) though Sharma et al. report a diagnostic accuracy of 83%(19) and Romero-Gomez et al. 79%(20) when compared to other neuropsychometric tests like computer-based, evoked potentials, NCT and PHES. Furthermore, a CFF <38 Hz predicts the risk of developing overt HE, correlating with Child-Pugh score, though not survival.(20)

PATHOGENESIS OF HE

Current paradigm

Though many factors have been implicated in the pathogenesis of HE, it is the interplay between ammonia, inflammatory responses and auto-regulation of cerebral haemodynamics (the 'multiple-hit' hypotheses) that appears most important and will be elucidated in greater depth in later chapters.

Ammonia: Arterial ammonia level (at admission) is an independent predictor of outcome(21) and a plasma ammonia level >150umol/L is associated with brain herniation,(22) with recent studies suggesting a linear correlation between arterial ammonia level and HE grade.(21, 23) *Post mortem* brain tissue from patients with hepatic coma and animal models of liver failure,(24) suggest that ammonia may be related to the development HE by propagating astrocyte swelling and cerebral oedema. In liver failure, progressive ammonia uptake in astrocytes, increases levels

of the osmolyte glutamine (catalysed by glutamine synthetase) with accumulation of water due the osmotic imbalance.(2) To better understand the role of ammonia in HE, one needs to understand interorgan ammonia & amino acid metabolism which will be discussed in chapter 1.2.

Inflammation/infection: Infection is detected in 80% of patients with acute liver injury(25) and its presence at admission is a predictor for worsening HE.(26) Development of sepsis may trigger decompensation of cirrhosis with progression to renal failure, encephalopathy, and gastrointestinal bleeding, with reduced survival. However, the associated proinflammatory cytokine release(27) is as important as the microbial pathogen itself. The term 'systemic inflammatory response syndrome' (SIRS) refers to the clinical manifestation of inflammation and describes a dysregulated host inflammatory response whether triggered by infection or not. Aseptic inflammation is just as implicit to the natural history of acute liver injury (e.g. drug induced ALF), and SIRS (in the absence of infection) is as significant to progression of encephalopathy as is SIRS triggered by infection.(28) However, it is plausible that in such studies worsening SIRS and encephalopathy are related to undetected infection, therefore establishing the presence of infection is paramount. Of particular concern in these patients is SBP that is diagnosed in 8-30% of hospitalised cirrhotic patients presenting with ascites and usually occurs in the absence of a primary focus of intra-abdominal infection. It arises by haematogenous spread of translocated intestinal flora via mesenteric lymph nodes. Predisposing factors are; – overgrowth of jejunal gram-negative aerobic bacilli and reduced intestinal barrier function and blood flow. In a patient with acute liver injury and uncontrolled intracranial hypertension, who underwent an emergency hepatectomy for critical hepatic necrosis (as a bridge to successful orthotopic liver transplant

(OLT), removal of necrotic hepatic tissue resulted in a sharp and sustained reduction in the circulating proinflammatory cytokine concentration and stabilisation of both systemic and cerebral haemodynamics independent of arterial ammonia.(29) This alludes to a relationship between systemically derived proinflammatory cytokines and cerebral blood flow modulation in the pathogenesis of intracranial hypertension in patients with acute liver injury.

***Cerebral blood flow modulation**:* In ALF, cerebral hyperaemia often develops, especially in those with malignant intracranial hypertension.(30) Even with medical treatment, malignant hypertension precedes death which can only be treated by orthotopic liver transplantation (OLT).(30) With the 2-3x increased blood-brain barrier permeability and cerebral metabolic rate for ammonia with HE,(31) elevated cerebral blood volume, besides increasing intracranial pressure, may serve to promote osmotic movement of water across the blood brain barrier predisposing to cerebral herniation.

Other pathogenic factors

There are a number of notable factors that are associated with progression of HE. However, their role appears to be intertwined with their interaction with the effect of ammonia on astrocytes. Such secondary factors, which modulate the role of the three principal pathogenic factors of HE – ammonia, inflammation and disrupted cerebral haemodynamic autoregulation, and worth a mention, are electrolyte imbalance and alterations in receptor concentrations.

Electrolyte imbalance: Anecdotal evidence from patients with ACLF, suggests that hyponatraemia is implicit to the progression to cerebral oedema.(32) A recent study

using ^{1}H-magnetic resonance spectroscopy (MRS), suggests that patients with hyponatraemia have significantly lower levels of the organic osmolyte *myo*-inositol(33) which would normally compensate for the rising intracellular glutamine seen with acute liver injury; other intracellular osmolytes are important (e.g. Taurine). Hypokalaemia increases renal ammonia production,(34) and if co-exists with metabolic alkalosis can potentially increase brain ammonia uptake by promoting formation of ammonia ($NH3^{+}$) which crosses the blood-brain barrier, from ammonium ($NH4^{+}$), a charged particle which doesn't. However, as discussed later (chapter 1.4), metabolic acidosis triggers increased renal ammonia production and relative increase in excretion.

Neurotransmitters/receptors: Metabolic and degenerative brain diseases characteristically demonstrate disorders of specific neurotransmission pathways. HE is no exception, with notable effects on glutamine, monamine, serotonin (5-HT), opiate, catecholamine pathways and γ-Aminobutyric acid (GABA), the chief inhibitory neurotransmitter in the central nervous system. GABAergic neurotransmission is one of the most investigated pathways over the last two decades, with reported "increased GABAergic tone" in HE.(35) This is suggested by a number of important observations. Firstly allosteric agonists of the GABA-A receptor complex (GRC) show similar visual evoked response potentials to rabbits with galactosamine-induced fulminant hepatic failure (FHF). Also, the activity of Purkinje neurons are more depressed by GRC modulator compounds in FHF rabbits compared to controls.(36) Also in patients with HE, Flumazenil - a highly selective benzodiazepine antagonist at the GRC, ameliorates neurobehavioural symptoms and EEG activity, suggesting its beneficial effect is not due to antagonist action on blood-borne endogenous benzodiazepines (see chapter 4). Proposed

pathophysiological mechanisms for this increased GABAergic tone includes increased brain GABA (due to increased uptake through altered barrier permeability), alteration in GRC integrity and/or increased endogenous GRC modulators such as benzodiazepines and neurosteroids (potent GRC agonists). However recent studies suggest that only increased endogenous brain agonists are likely to be important; with no significant alterations of either GABA content,(37, 38) or GRC integrity. GABA activates the postsynaptic GRC, a ligand-gated chloride channel, which then allows chloride to enter and inhibit the postsynaptic neuron. Barbiturates, benzodiazepines and neurosteroids (e.g. tetrahydoprogesterone (THP) also known as allopregnanolone and tetrahydrodeoxycorticosterone (THDOC)) all bind at the GRC; neurosteroids enhancing chloride uptake and positively influencing GABA and benzodiazepine agonist GRC binding.(39) It appears however, that only neurosteroids concentrations(40) and not benzodiazepine levels are increased in HE and therefore important to its pathogenesis. However, although such neurotransmitter pathways show promise, the interaction with other key pathogenic factors of HE like ammonia and electrolyte imbalance are likely significant, making it hard to elucidate the true role of specific neurotransmitters in this condition. Therefore as yet there are few therapeutic interventions that target the relevant neurotransmitters, which have proven to be of benefit outside of laboratory and animal experimentation, and though further study is needed, the role of neurosteroids was not investigated in this thesis.

Nutritional factors: Patients with cirrhosis (especially if alcohol-induced), usually have a poor nutritional reserve due to anorexia, poor diet, malabsorption, and altered metabolic state. Furthermore, hospitalised patients are often hypermetabolic and hypercatabolic, worsened by complications such as gastrointestinal bleeding,

continued anorexia and fasting for tests. Given concerns of nitrogen loading with high calorie intake, clinicians previously advised dietary protein restriction in liver failure to prevent a rise in blood ammonia levels. However, this has the potential to lead to a worsening negative nitrogen balance, which aside from limiting energy production will reduce muscle mass that may alter the potential of skeletal muscle glutamine synthetase to buffer ammonia. Recently, the 'European Society for Parenteral and Enteral Nutrition' consensus review(41, 42) recommended a normal/higher supply of dietary proteins to achieve nitrogen balance, which can be tolerated without risk of HE. However, infrequently in certain cases where the patient has persistently high circulating ammonia and intracranial hypertension and oedema despite other interventions, short-term low protein feeds (e.g. short-term glucose infusions) are occasionally used to provide calories (energy), without nitrogenous compounds. In advanced cirrhosis, there is also evidence of an increase in aromatic amino acids and reduction in branched-chain amino acids (BCAAs) resulting in effects on neurotransmitter synthesis, associated with HE; providing a rational for preferentially supplementing dietary BCAAs.(43, 44)

In conclusion, despite a number of different causal factors in the development of HE, the role of ammonia and precipitating factors in disease progression continues to be key to its pathogenesis and influence current 'best treatment', which will be outlined in chapter 1.4. There is also a need for better designed and powered randomised control studies, if not metanalysis, assessing the therapeutic benefit of interventions targeting HE.

Table 1: Western Haven criteria

Stage	Consciousness	Intellect and Behaviour	Neurological Findings	EEG findings
0	Normal	Normal	Normal examination; impaired psychomotor testing	Normal, slowing (4-6Hz)
1	Mild lack of awareness, personality change, day/night reversal	Shortened attention span; impaired addition or subtraction	Mild asterixis or tremor, mild incoordination	Slowing
2	Lethargic, inappropriate behaviour	Disoriented	Obvious asterixis; slurred speech, abnormal reflexes	Slowing, triphasic
3	Asleep, somnolent but rousable	Gross disorientation; bizarre behaviour; loss of meaningful communication	Muscular rigidity and clonus, hyperreflexia, abnormal reflexes	Slowing, triphasic
4	Coma	Coma	Decerebrate posturing	Very slow (2-3Hz), delta

Table adapted from that originally published by Conn HO. Trail-making and number-connection tests in the assessment of mental state in portal systemic encephalopathy. Am J Dig Dis 1977;22:541-550.

Table 2: Differential diagnosis

Metabolic	Toxic	CNS
Glycaemic control - hyper	Alcohol - intoxification	Encephalitis
- hypo	- withdrawal	Meningitis
Calcaemic control - hyper	Drugs - prescribed	Abscess
- hypo	- illicit	Trauma
Hypokalaemia	Carbon monoxide narcosis	Tumour
Hypoxia		Bleed
Uraemia		Infarction

Table 3: HE precipitants

Precipitating Factors In Hepatic Encephalopathy
Increased nitrogen
• Constipation
• Gastrointestinal bleeding
• Azotaemia/uraemia
• Excessive dietary protein
Electrolyte imbalance
• Hypokalaemia
• Hypoglycaemia
• Hypothyroidism
• Hypoxia
• Hypotension
• Dehydration
• Metabolic alkalosis/acidosis
Drugs
• Narcotics sedatives
• Tranquilizers
• Sedatives
Miscellaneous
• Infection (e.g. subacute bacterial peritonitis)
• Surgery
• Anaemia
• Progressive liver disease
• Superimposed liver disease (e.g. hepatoma)
• Vascular occlusion
• TIPS and surgical shunt insertion

Chapter 1.2

Hepatic Encephalopathy:

Interorgan Ammonia and Amino Acid Metabolism in HE

Introduction

The impact of liver disease on neuropsychological function, termed hepatic encephalopathy (HE), is incumbent on the accumulation of ammonia.(45) With liver failure, aside from urea cycle disruption and portal-systemic shunting, ammonia homeostasis is dependent on differential changes within a number of key organs, amino acids (glutamine metabolism(46)) and enzymes. However, aside from liver transplantation, recent meta-analysis suggest that current clinical strategies which primarily target lowering ammonia, fail to make a clinical impact on HE.(47, 48) The shortfall in donor organs for transplantation dictates a need for new therapeutic interventions to correct hyperammonaemia. This chapter highlights interorgan ammonia and amino acid metabolism pertinent to liver disease and hyperammonemic states and future concepts based on this understanding which may lead to more targeted therapy.

CELLULAR AMMONIA AND AMINO ACID METABOLISM

Dietary intake and production: Nitrogen is necessary for cellular structure and energy. Humans assimilate reduced nitrogenous compounds as dietary protein, free amino acids, and the gas- ammonia, derived from the splitting of urea (and possibly amino acids) by 'urease'-producing intestinal bacteria; with their relative importance in intestinal ammonia metabolism to be discussed later.

Chemical properties: In the body ammonia (NH_3) is co-existent with its charged form ammonium (NH_4^+), with the time to equilibrium of- '$NH_3 + H^+ \leftrightarrows NH_4$' (milliseconds), dependent on pH.(49) At a physiological pH, 98% of ammonia exists

as NH_4^+, but as NH_3 is the main form transported across biological membranes, in this review we refer to NH_3/NH_4^+ as simply, '*ammonia'*.

Plasma transport: Ammonia is hydrophilic and easily transported in plasma unlike albumin-bound hydrophobic toxins (e.g. bilirubin, bile and fatty acids) that accumulate in liver failure. In health, ammonia transport is tightly regulated to maintain low plasma concentrations (normal range 10-40 μmol/L). In several organs (e.g. intestinal or kidney) ammonialevels are the mmol/L range.

Transmembrane movement: Ammonia's moderate lipid solubility should limit membrane permeability, but as most membranes are very thin, ammonia permeability is good; though with highly lipid based membranes it maybe impermeable. Charge is also important, with metabolic alkalosis increasing the conversion of NH_4^+ to NH_3, serving to improve ammonia membrane permeability. However, within the cell negatively charged mitochondria show a very poor permeability to NH_3 despite a high pH, but do display a high permeability to NH_4^+.(50) Transmembrane ammonia transport involves a number of constitutive ion channels (discussed later), influenced by certain transporters (e.g. Aquaporins). Aquaporins (AQP) are bi-directional water channels that may increase the permeability of the cell to ammonia (e.g. AQP3, AQP7, AQP8 and AQP9).(51-53) Ammonia's membrane movement is pH-dependent and driven by the NH_3 gradient(54) which like the osmotic gradient is bidirectional. Given there wide distribution and variable permeability properties it is possible that differential AQP expression may play a role in cellular and whole body ammonia homeostasis, independent of their effect on water transport. Recently, the erythroid 'Rhesus family' transmembrane proteins,(55-57) were found on epithelial cells of the

intestinal, kidney and liver,(58) influencing ammonia transport in these organs. However, their precise role in whole body ammonia metabolism remains unclear.

INTERORGAN AMMONIA AND AMINO ACID METABOLISM

Relationship between ammonia, glutamine and glutamate

Whole body ammonia metabolism is dependent on the localisation, activity and differential concentrations of certain key enzymes common to all organs.(59) Glutamine Synthetase (GS) converts ammonia and glutamate to glutamine [Glutamate + adenosine tri-phosphate (ATP) + NH_3 → Glutamine + adenosine di-phosphate (ADP) + phosphate] expending 1 ATP for every molecule of ammonia consumed. Phosphate-activated glutaminase (PAG) carries out the reciprocal reaction - glutamine to glutamate and ammonia. Glutamine is a non-essential amino acid (five-carbon chain and two nitrogen residues), abundant in protein and constitutes 50% of the whole body free amino acid pool.(60) Glutamine and glutamate are therefore central to organ and whole body nitrogen balance by serving as both nitrogen donor and acceptor; with glutamine both a sink for excess ammonia (GS), or its source (PAG).(61) It is the location, expression and activity of these key enzymes that impact on the ammonia and amino acid metabolic flux of different organs in both health and disease which influences circulating ammonia levels.

Interorgan ammonia metabolism

There are multiple organs involved in whole body ammonia and amino acid metabolism:

Intestines: Feeding increases intestinal ammonia generation, with dietary protein yielding differential ammoniagenic potential(62) (meats > dairy(63) > vegetable protein(64)), possibly influenced by carbohydrate.(65) In addition to dietary protein and intestinal bacterial ammonia production, studies on post-absorptive healthy animals show that 50% of intestinal ammonia is from amino acids derived from its blood supply (Figure 1).(61, 66, 67) This is mainly from circulating glutamine (the enterocytes main energy source) taken up and converted by phosphate-activated glutaminase (PAG), to ammonia and glutamate (subsequently transamidated to alanine(68, 69)), before its release into the mesenteric vein. In both humans and rats by measuring enzyme activities in mucosal biopsies from different intestinal sites, about 80% of intestinal PAG is found in the small bowel and 20% in the large bowel,(70, 71) to a greater extent equating to their relative activities. This is further suggested by the correlation between PAG activity and MHE. Therefore, due to high relative abundance of PAG, the intestines are a major glutamine-consuming and ammonia-producing organ,(68, 69) as suggested by substantial intestinal ammonia production in germ free rats, or hyperammonaemia in germ-free hepatectomised rats**.**(72) Such observations also serve to highlight that intestinal bacterial ammonia production is not the predominant contributor to systemic ammonia as historically thought. Differences in arterial-venous ammonia from gastrointestinal (GI) malignancy patients undergoing elective abdominal surgery supports animal data, with glutamine extraction (24% Jejunum, 9% Ileum and 8% colon), associated with jejunal and ileal ammonia release.(73) The colon provides the remaining intestinal ammonia (which in dogs is derived from 9% glutamine, 42% arterial urea and 49% from luminal bacterial breakdown products, glucose, short-chain fatty acids and ketones).(66) Therefore in the post-absorptive state, around 50% of intestinal ammonia arises directly from dietary nitrogen and 50% from

circulating amino acids; with equal contribution from the small bowel (with its predominant circulating amino acid conversion) and large bowel (with its predominant bacterial amino acid and urea breakdown). Although as discussed later, the small bowel may become increasingly important with progressive liver injury.(74)

Liver: A typical diet contains ~100grams protein/day (25% glutamate–producing amino acids - glutamine, ornithine, proline, histidine, arginine and glutamate); the bulk metabolised by the liver. Excessive dietary nitrogen must be either excreted, or converted to a non-toxic form. This is achieved in the compartmentalised periportal and perivenous hepatocytes of the liver acinus. Firstly these nitrogenous compounds (mainly as glutamine of ammonia) need to get into the hepatocytes. Ammonia is readily diffusible and assisted by a number of transporters (see earlier). Glutamine movement is aided by the transport protein 'sodium-coupled neutral amino acid transporter' (SNAT3)(75) (found in liver, kidney, muscle and astrocytes), which is important for both glutamine uptake in periportal cells and glutamine release by perivenous cells; due to bi-directional properties related to its ability to counter-transport H^+.(75, 76)

Periportal hepatocytes: contain the *'hepatic urea cycle'* (Figure 2) that culminates in the conversion of ammonia (produced by oxidative deamination) to urea, the major end-product of nitrogen metabolism. One mole of urea removes 2 moles of waste nitrogen but requires 4 moles of ATP (2 ATP per ammonia); 15 mgs of total soluble ammonia, given production of 1 gram of urea [$2\ NH_3 + CO_2 + 4ATP$ ---> *urea* [$(NH2)_2CO$] + $H_2O + 4ADP$]. The 'urea cycle' is not the only route for ammonia detoxification, as bicarbonate (HCO_3^-), rather than CO_2 may be involved; impacting in pH metabolism (see later). Periportal hepatocytes are highly abundant and a prominent site for not only urea cycle enzymes (e.g. carbamoyl-phosphate

synthetase (CPT), the 1st step and rate limiting enzyme of the cycle), but also PAG and alanine-/aspartate aminotransferases (ALT/AST). Liver PAG has a low-affinity and high capacity for its product (ammonia),(46) which is also activated by NH_3 (but not NH_4^+). PAG is also critical to urea cycle, by providing intramitochondrial glutamate for N-acetylglutamate synthesis, activating CPT. Therefore, the bigger the supply of intestinal ammonia (and glutamine), the bigger its turnover to urea.(77, 78) Alanine is another important source for urea via periportal ALT and AST. Collectively, urea synthesis derives from - 33% portal ammonia, 6-13% portal glutamine, 20% mitochondrial glutamine and 33-40% others (e.g. portal and hepatic alanine, and hepatic ammonia and glutamine via various enzymes).(79)

Perivenous hepatocytes: These cells surrounding terminal venules, make-up only 7% of hepatocytes and via hugely abundant GS (high affinity, but low capacity for ammonia(46)) convert ammonia to glutamine.(80) Therefore, if any ammonia escapes periportal hepatocytes, it can be scavenged and detoxified by perivenous hepatocytes. GS and PAG act in concert to accommodate rapid changes in systemic ammonia levels from one of glutamine uptake to glutamine release.(81-83) Hepatic glutamine metabolism, in concert with urea synthesis, is therefore very important in systemic ammonia detoxification due to the unique regulatory properties of hepatic glutaminase, acinar compartmentalisation ('zonation') of urea and glutamine synthesis, and 'intracellular glutamine cycling'(78) between periportal and perivenous hepatocytes. Metabolic zonation, the spatial organization of various metabolic pathways and functions, forms the basis for the efficient adaptation of liver metabolism to the different nutritional requirements of the whole organism in different metabolic states. As such, in the post-absorptive state with a normal healthy liver, hyperammonaemia should never occur due to the capacity of the hepatic urea cycle and intercellular glutamine cycling (Figure 3).

A further ability of the urea cycle is pH homeostasis. Portal ammonium ions can also be converted into urea, with the simultaneous involvement of bicarbonate (HCO_3^-) rather than CO_2 [NH_3 + HCO_3^- → urea]. This is a significant pathway at time of metabolic acidosis helping to maintain pH homeostasis, as unlike glutamine synthesis, urea synthesis removes bicarbonate; this leads to a change from urea synthesis to glutamine synthesis during acidosis, to conserve bicarbonate.(78, 84)

The Kreb's cycle is the primary metabolic pathway for cellular energy production, converting carbohydrates, fats and proteins to energy, with CO_2 and water as waste. The urea cycle is closely linked to the Kreb's cycle deriving one of its nitrogens through transamination of oxalacetate to form asparate and returns fumarate to that cycle. This allows amino acids formed by the urea cycle to be used as a source of fuel in the Kreb's cycle; which is important as energy is expended through the urea cycle (~20% of the energy derived from metabolism of gluconeogenic amino acids). Ammonia is quickly combined with α-ketoglutarate to form glutamate (glutamate dehydrogenase (GDH)) and glutamine (GS). The NH_2 groups can then be moved on to other recycled carbon skeletons to form new amino acids by transamination and transamidation by vitamin-B6-dependent ALT/AST (e.g. keto acids, pyruvate and oxaloacetate). Relative substrate levels largely determine the reaction direction of transaminases (as the equilibrium constant approaches 1). The only amino acids that are not formed during this process are the diet-dependent essential amino acids - threonine and lysine. As GDH is NADPH-dependent, cellular energy status critically regulates hepatic ammonia and amino acid metabolism. In low energy states proteogenesis is limited, promoting the reverse instead with glutamate converted to ammonia and oxidizable cycle intermediates for energy production.

Changes in pH also directly influence the hepatic enzymes, with acidosis also reducing the rate of glutamate metabolism by affecting mitochondrial PAG and GDH flux,(85) while as mentioned earlier, stimulating glutamine synthesis and recycling. Therefore acidosis directly effects ureagenesis by effecting PAG-dependent glutamate metabolism via its influence on nitrogen provision for carbamyl phosphate and N-acetylglutamate synthesis;(85) with alkalosis having the converse effect.

Kidney: Urinary ammonia excretion involves tightly regulated mechanisms such as tubular urine flow, apical/basolateral ion exchangers (e.g. Na^+-K^+-NH_4^+-ATPase) and ammonia counter-current system. In the post-absorptive state, glutamine is the main substrate for renal ammoniagenesis,(86) but unlike other organs kidney-PAG is strongly inhibited by glutamate (its product), not ammonia. Ammonia synthesised by proximal tubular cells is excreted into tubular fluid and quickly reabsorbed by the medullary thick ascending limb (MTAL) (Figure 4). There it accumulates in the interstitium before excreted as NH_4^+ (due to the effect of urinary acidification) in the medullary collecting ducts.

In the post-absorptive state of humans, urinary ammonia excretion reflects the renal excretion rate(86) and match ammonia circulatory release.(86, 87) In post-absorptive animals (e.g. dogs,(88) pigs(89) and rats(90)) 70% of renal ammonia is released into the circulation (renal vein) with only 30% excreted in urine. Owing to compensatory GS and glutaminase activity with changing arterial ammonia delivery, the kidney can become a net producer or excretor of ammonia,(91) as evident by an increase in ammonia renal excretion with elevated plasma ammonia in healthy volunteers administered ammonium chloride.(86) Yet under certain conditions (e.g. changes in acid-base balance) this can shift to net ammonia production.

Acid-base balance: In response to acute metabolic acidosis, within the kidney proximal tubular cells take up glutamine and due to increased PAG activity form ammonia and glutamate.(85) Glutamate is then either released into the renal vein, transamidated to form alanine (hepatic energy source), or converted to α-ketoglutarate and ammonia (by GDH).(92) Alpha-ketoglutarate can then be metabolised to either lactate and glucose (for release into the renal vein),(93) or pyruvate for oxidization to CO_2 in the TCA cycle. Such decarboxylation of α-ketoglutarate causes net circulating bicarbonate synthesis, to compensate for the acidosis.(94) By conserving circulatory bases to compensate for acidosis, more ammonia can be excreted (≤70% of total renal ammonia production), at the expense of urea to conserve acid-base balance, reversing normal conditions while maintaining nitrogen excretion at a constant.(88, 95) The reverse may occur with alkalosis.

Hormonal control also has a role, with Angiotensin II dose dependently increasing renal ammoniagenesis by directly increasing MTAL absorption via its effect on Na^+-K^+-NH_4^*-CL^-. Via such ammonia ion-exchangers, renal ammonia transport is also differentially sensitive to certain diuretic therapies (e.g. Frusemide).

Muscle: Although the GS activity of skeletal muscle is significantly greater than of competing glutaminase activity,(96) GS activity in skeletal muscle is still low;(97) but by virtue of muscles mass it can impact greatly on interorgan ammonia metabolism. In healthy volunteers, one study suggested an estimated 50% arterial ^{13}N-ammonia extraction from skeletal thigh muscle.(31) However, other studies demonstrate a zero arterial-venous difference across forearm and leg suggesting an absence of muscle ammonia uptake.(97-100) which is a more robust measure of organ metabolism as extraction does not truly reflect a net metabolic

consumption/production as this is dependent on organ flow. Skeletal muscle ammonia production also provides an additional route for nitrogen (and carbon) transport from muscle to liver, with muscle pyruvate transaminated to alanine and then transported to the liver for conversion to ammonia (by ALT). Once in the liver, alanine (with α-ketoglutarate) is converted to pyruvate and glutamate (also by ALT). The generated liver pyruvate can be incorporated into the Kreb's cycle by pyruvate dehydrogenase (PDH), oxidatively decarboxylated to acetyl coA and thus used as a substrate for gluconeogenesis which can be recycled back to muscle. The glutamate produced by this reaction also allows for the formation of ammonia (and glutamine) by liver PAG and later urea; with α-ketoglutarate recycled from glutamine dehydrogenase (GDH). This 'glucose-alanine cycle' (Figure 5) allows skeletal muscle to eliminate nitrogen while replenishing its energy supply. Under conditions of fasting, the glucose-alanine cycle was originally thought to maintain glucose homoeostasis when exogenous supplies of energy were limited.(101) But given concerns over the relative significance of carbon homeostasis between glucose, lactate and alanine in the muscle,(102, 103) recent evidence suggests that it is more important in maintaining nitrogen transport,(104) with alanine a major source of urea.(105)

Brain: Ammonia is important for the regulation of glial cell metabolism (a signaling function). In health, ammonia readily traverses the blood brain barrier, with positive arterial-venous differences suggesting net brain ammonia uptake; with the rate of arterial ammonia clearance directly correlating to arterial concentration. Though some studies suggest little/no net ammonia uptake,(99, 106) arterial extraction (^{13}N-ammonia) maybe as high as 47% within gray matter (astrocytes).(31) Astrocytes (20% of brain cells) contain 80% of brain GS, whereas neurons contain

20% (and nearly all brain Glutaminase).(107, 108) Astrocyte GS preferentially takes up ammonia and glutamate to form glutamine, which can then be transferred to neurons for deamination to re-form glutamate and GABA (important neurotransmitters). Furthermore glutamate can be converted to alpha-ketoglutarate and lactate and transported to neurons as a source of energy, though changes in intracellular pH and K^+ effect the regulation of these glial-neuronal metabolic processes.(85) Therefore in health, the brain is a significant organ for ammonia utilisation and detoxification; comparable to skeletal muscle.(31) However, amino acids involved in neurotransmission must be limited to reduce neurotoxicity. In glial cells, glutamate uptake is dependent on excitatory amino acid transporter (EAAT-2) and glutamate transporter (GLT-1) and in neurons on excitatory amino acid carrier (EAAC) and EAAT-3. If extracellular glutamate accumulates (usually kept within micromolar range) it may lead to toxic N-methyl-D-aspartate (NMDA) receptor activation and neuronal death. Intracellular glutamate (at milimolar levels), is actively taken up (without ATP) against an electrical gradient by co-transport of ions moving down their electrochemical gradients (3 Na^+ and one H^+ enter, while one K^+ is transported out of the cell), as glutamate is slightly negatively charged as is the inside of the cell.

Lung: Lung parenchyma also possess PAG and GS,(59) but although there is the potential to play a role in whole body ammonia metabolism, recent evidence from interorgan ammonia and amino acid trafficking in pigs,(109) suggest that in health the lung has little net effect.

Heart: As the heart is muscle it has a significant amount of GS(83) and also contains PAG(96) and therefore a possible role in ammonia metabolism, but evidence is limited.

AMMONIA IN DISEASE

Evidence of a role for ammonia in liver disease

The importance of ammonia with liver disease was first identified by Nencki & Pavlov (1896) who demonstrated that hyperammonaemia induced neurobehavioral change in portacaval shunted (PCS) dogs.(110) This hepatic encephalopathy (HE), encompasses a spectrum of mental disturbances that range from sub-clinical to coma and death.(6) Since, innumerable studies provide the consensus view that ammonia is central to the pathogenesis of HE. In liver failure, the presence of HE defines temporal classification of liver failure. Arterial levels best prognosticate circulating ammonia, correlating to HE severity;(23) though venous sampling is adequate. In acute liver failure (ALF), arterial ammonia levels of >150umol/L predict a poor outcome(22) and correlate with increased ICP and cerebral oedema.(21, 22, 111) This is likely to be related to increased brain delivery and uptake of ammonia associated with hyperammonaemia highlighting the importance of interorgan ammonia metabolism.

Inherited enzyme deficiencies

In addition to liver failure, as there is no alternative pathway for urea synthesis, inherited disorders of the hepatic urea cycle may cause profound accumulation of toxic by-products (e.g. CPT), with hyperammonaemia, mental retardation and even death, though this will not be further discussed in this review.

Liver failure: altered interorgan ammonia metabolism (Figure 6)

Animal models of liver failure show a widespread disturbance in ammonia and glutamine metabolism,(109, 112) supporting similar, though limited data (owing to complexity) from clinical studies.(113-117) But caution is necessary in correlating findings from animal models (with variable extent, type and duration of injury) to human physiology and disease. Furthermore, most studies utilise arterial-venous differences across organs. Yet, this fails to reflect the rate of ammonia production/consumption, referred to as flux, which is dependent on organ flow. However, organ flux measurements are necessary to enable quantification of metabolism and comparison of individual organs.

Intestines: Cirrhosis is associated with a four-fold increase in intestinal PAG activity in the small bowel,(74) pointing to an increase in the relative importance of small bowel ammoniagenesis (derived from circulating amino acids) with liver injury.(74) In stable patients with cirrhosis and a transjugular intrahepatic portosystemic shunt (TIPS), there is net intestinal ammonia production (though limited) which directly correlates with glutamine uptake.(118) In patients with cirrhosis and a TIPS, made hyperammonemic by either a simulated bleed (amino acid solution mimicking haemoglobin) or acute variceal GI bleeding, there was no sign of increased net intestinal ammonia production, no efflux from the splanchnic circulation or muscle uptake, but instead a significant increase in renal production (6-fold).(115) In PCS rats, intestinal ammonia production and stiochiometric glutamine consumption was no different to the post-absorptive state.(119) In pigs, ALF did not provoke net intestinal ammonia production.(109) In PCS rats, induction of ALF with hepatic artery ligation (HAL) caused an early and slight increase in

ammonia production and glutamine consumption, though at 4hrs ammonia remained elevated despite a fall in glutamine consumption.(119) A loss of stiochiometric ammonia production to glutamine consumption suggests that with liver failure the intestines contribution to the hyperammonemic state is mainly due to portacaval shunting and not to changes in the intestines ability to metabolise ammonia and amino acids.(109) This is possibly due to the saturation of PAG, with rising arterial glutamine (8-fold) reflecting reduced consumption (or muscle release), as arterial ammonia eventually plateaus;(120, 121) as with earlier *in vitro* data.(68)

Hyperammonaemia due to the ammoniagenic blood protein meal of a bleed, is possibly related to an absence of the branched-chain amino acid - isoleucine.(122-126) In patients with cirrhosis given a simulated bleed, administration of intravenous isoleucine improved hepatic and muscle protein synthesis (e.g. net phenylalanine incorporation) without any significant effect on the kidneys or intestines.(117) Isoleucine may also be cerebroprotective against HE, bypassing ammonia's inhibition of the TCA cycle (alpha-ketoglutarate dehydrogenase) through provision of acetylCoA and succinylCoA. However, in the absence of bleeding, administration of intravenous isoleucine to correct HE in ALF rats was shown to have no effect on coma stage, cerebral oedema, brain lactate or plasma ammonia level.(127)

Liver: Hepatocyte injury limits ammonia detoxification by reducing periportal urea and perivenous glutamine synthesis,(82, 128) but there is usually enough ammonia detoxifying capacity to remove it all from the portal vein and hepatic artery until advanced liver failure.(22) With portal-systemic shunting ammonia detoxification can be further compromised as it may account for 50% of portal flow,(129, 130)

and as much as 93% in TIPS patients.(129, 130) In response to the rising ammonia of liver failure, despite compensatory increases in periportal glutaminase activity (≤6-fold) and ammonia fed into the 1st step of the urea cycle (CPT),(82) failure to compensate increases the amount of ammonia passed through to the terminal venules.(84) As perivenous GS capacity also becomes swamped and fails to scavenge ammonia, it leaches into the hepatic vein with post-absorptive levels of 40-60μM in cirrhosis, 70-90μM in acute-on-chronic liver failure (ACLF) and 200-240μM in ALF; correlating with arterial ammonia concentrations.(22) The lower levels with stable cirrhosis (minimal ammonia flux across the hepato-splanchnic area), suggests that the liver is still able to remove the majority of ammonia from the portal vein and hepatic artery until quite late on. Even with advanced disease demonstrable ammonia removal may be somewhat masked by even significant portal-systemic shunting.(22)

Muscle: Skeletal muscle ammonia uptake is correlated to arterial levels at various stages of ALF(99, 100) and cirrhosis.(97, 98) On a background of hyperammonaemia, specific organs (muscle and brain), due to their predominant GS activity, try to compensate by switching to net ammonia consumption. In ALF patients with advanced HE, skeletal muscle consumes ammonia (100nmol/100g/min) and glutamate with the stiochiometric release of glutamine.(113) However, with the catabolic state that is ALF, such increased glutamine release may impart reflect muscle catabolism and resultant amino acid production (10-fold(113)), rather than ammonia conversion from increased GS expression and activity(131, 132)). In cirrhotic patients with hyperammonaemia who underwent TIPS for gastrointestinal bleeding, skeletal muscle was also the main site of ammonia removal.(115, 133) But, hyperammonaemia does not usually

occur in stable cirrhotics with normal muscle mass,(99) only in those with significant muscle wasting,(97-100, 106) due to reduced ammonia extraction;(97) this highlights the need to maintain adequate muscle mass. Skeletal muscle mass therefore, provides an alternative therapeutic target for ammonia detoxification(134) as supplying ornithine (providing 2 glutamate) can theoretically buffer the hyperammonemic state of liver failure by GS converting ammonia and glutamate, with glutamine efflux.(132)

Early clinical studies reported increased circulating glutamate with liver failure,(135-137) but recent evidence tends to confirm the converse – a circulating glutamate deficiency;(113, 116, 138) due to a reduction in hepatic synthesis and/or conversion of stores to glutamine. However, in ALF (PCS + HAL) devascularised pigs, despite an initially raised arterial glutamine at 2 hours, levels eventually fell (8-fold), possibly due to a fall in glutamate provision.(109) In contrast to clinical studies, in the ALF devascularised pig model there was no net skeletal muscle ammonia uptake 6 hours post ALF, despite initially increased at 2 hours.(109) Absent muscle ammonia uptake was also reported in PCS rats with acute liver ischaemia with no net glutamine efflux despite increased muscle glutamine levels.(120) Contrasting results between animal experiments and clinical studies may only reflect differences in species physiology and/or type and extent of liver injury. Also, skeletal muscle ammonia uptake and glutamine release does not necessarily lead to net whole body ammonia detoxification as muscle-derived glutamine can be taken up by the splanchnic region or kidneys and converted to ammonia for release into the circulation.

Kidney: As discussed earlier in this chapter, Glutamine is the main substrate for renal ammoniagenesis. In the PCT, glutamine is converted to ammonia and

glutamate by PAG, with GDH further converting glutamate to ammonia. Ammonia (NH_3) is excreted into tubular fluid by non-ionic diffusion (pH gradient) and ammonium (NH_4^+) via ionic diffusion (electrical gradients). Accumulated ammonia in the medullary interstitium of the ascending limb of the loop of Henle is quickly reabsorbed by the DCT of the MTAL via co-transporters (blocked by loop diuretics). This allows for urinary acidification and buffering, with the Renin-angiotension-aldosterone system (RAAS) dose-dependently influencing renal ammoniagenesis by directly increasing MTAL absorption via co-transporters effects. In health, 70% of renal ammonia is released into the circulation (renal vein) with only 30% excreted in urine. However, though ammonia excretion is not directly correlated to plasma levels,(98) with advancing liver disease the kidney becomes an important source of ammonia.(109, 115, 139) Autoregulation maintains renal blood flow (RBF) and glomerular filtration over a wide range of blood pressures, but increased noradrenaline (NA) with renal syndromes of liver failure (e.g. HRS), reduces RBF and perfusion, or use of diuretic therapy(140) further reduces ammonia excretion.

Animal models allow study of the influence of renal metabolism on hyperammonaemia.(141-143) In PCS rats, in response to moderate hyperammonaemia (arterial levels ~ 250 μmol),(142) the kidneys seemingly adapt by increasing glutamine uptake and/or decreasing plasma release(115) to reverse the normal 30/70 urinary excretion/renal venous release ratio and cause a shift to ammonia excretion. However, in PCS rats 6-hrs post induction of ALF, severe hyperammonaemia (arterial levels ~ 950 μmol) was associated with a reduced ammonia excretion and net renal ammonia production.(142) Elevated renal ammonia release in this model was likely triggered by increased muscle glutamine production and renal uptake. Furthermore, in ALF pigs there is a significant decline in renal glutamine uptake and ammonia excretion in urine with time,(109) indicating that in

more advanced disease the ability of the kidney to compensate for hyperammonaemia by excreting ammonia is overrun.

Splanchnic vasodilation, associated with over-activated RAAS, is characteristic of cirrhosis.(114, 144) In patients with cirrhosis, correcting hypovolaemia may therefore increase renal ammonia excretion and reduces plasma ammonia(145) by reversing influence of an over-activated RAAS on renal perfusion. This is suggested by an improved renal ammonia excretion in patients with cirrhosis following emergency TIPS for variceal bleeding,(117) with a fall in renal ammonia circulatory release (to almost zero) at one hour post-TIPS. Limiting renal ammonia release into the circulation may be an early adaptive effect of TIPS, persisting as long as 25 months.(118) Collectively these observations support the presence of renal ammonia adaptation with early hyperammonaemia, which with worsening hyperammonaemia and renal dysfunction (e.g. diuretics, dehydration, HRS etc.) later switches to net renal ammonia production and circulatory release. However, the limited contribution of the kidney should be put in context compared to the impact of portosystemic shunting and failure of hepatic urea synthesis.

Brain: Brain delivery, extraction and uptake of ammonia increases in ALF;(31, 116, 146) correlating with arterial levels.(106) However, ammonia extraction data are variable, with some reporting extraction only in comatose patients, while others show 11%-15% extraction in the non-comatose. Ammonia detoxification (via the amidation of glutamate by GS) produces glutamine accumulation and thus osmotic stress,(2) - the *'ammonia-glutamine-brain swelling hypothesis'.* In ALF, brain glutamine correlates with arterial ammonia,(147) with around 66% of brain ammonia metabolised to glutamine and the rest pooling in the brain.(148) However, this brain pool does not occur with cirrhosis as plasma levels reach a

steady state. To maintain osmotic equilibrium as intracellular glutamine increases, astrocytes expel myo-inositol and other weaker osmolytes. However, due to the rapidity of ALF, dramatic shifts in ammonia outstrip compensatory mechanisms leading to oedema. In cirrhosis, there is some protection from intracranial hypertension and brain edema because of the more gradual increase in plasma ammonia concentration allowing time for a compensatory expulsion of weaker intracellular osmolytes,(149) though oedema may still occur.(150) However, one should stress that although never formally studied, net brain ammonia detoxifying capacity is minimal compared to skeletal muscle due to its mass.

Some argue that oxidative stress and free-radical production rather than intracellular glutamine are more closely associated with (MAPK-dependent) astrocyte swelling(151, 152) and worsening HE;(153, 154) explaining how antioxidants and hypothermia may limit oedema.(155-158) However, ammonia is still central as both are secondary to ammonia metabolism following glutamine breakdown by mitochondrial PAG.

In ALF, elevated brain ammonia may also negatively influence cerebral energy metabolism(38, 159, 160). Inefficient 'anaerobic' glucose breakdown (less ATP per glucose than through the Kreb's cycle) and direct inhibition of alpha-ketoglutarate dehydrogenase (the rate limiting step in the Kreb's cycle), increases lactate and alanine synthesis.(38, 161) Astrocyte glutamate and ammonia uptake is also dependent on ATP requiring Na^+-K^+-ATPase co-transporter;(162) though much of the uptake occurs along ionic gradients without the need for energy. Furthermore, high glutamate levels that occur with a disrupted 'malate–aspartate shuttle' may also impair energy metabolism.(163)

Neurotoxicity: In neuronal tissue, ammonia is converted to glutamate (via glutamate dehydrogenase), depleting the brain of a-ketoglutarate and so inhibit the

Kreb's cycle.(108, 164) The resulting fall in oxaloacetate reduces ATP availability.(38, 160) In the absence of aerobic oxidative phosphorylation and Kreb's cycle activity, cell damage and neural cell death occur. Neurotransmission is further impaired by depletion of glutamate stores (due to glutamine formation) and thus NMDA, GABA and benzodiazepine receptor activation. This could dampen inhibitory synapses (increasing excitability), or inhibit excitatory pathways.

Infection is observed in more than 80% of patients with ALF,(25) and frequently precipitates HE.(25, 26, 28, 153) In the brain this interaction may take place in astrocytes as they detoxify ammonia which may result in swelling(2), have an extensive inflammatory mediator repertoire, and regulate cerebral blood flow (possibly through an arachidonic acid and cyclooxygenase dependent pathway).(165) Thus, hyperammonaemia may 'activate' astrocytes 'unlocking' the blood-brain barrier, making them susceptible to endotoxaemia through a cyclooxygenase (COX)-dependent mechanism.(166) Taken together, disrupted ammonia, amino acid and cerebral energy metabolism influenced by inflammatory responses, effects neuronal and glial cell function with direct effects on glial cell osmotic gradients, cerebral blood flow and thus development of HE.

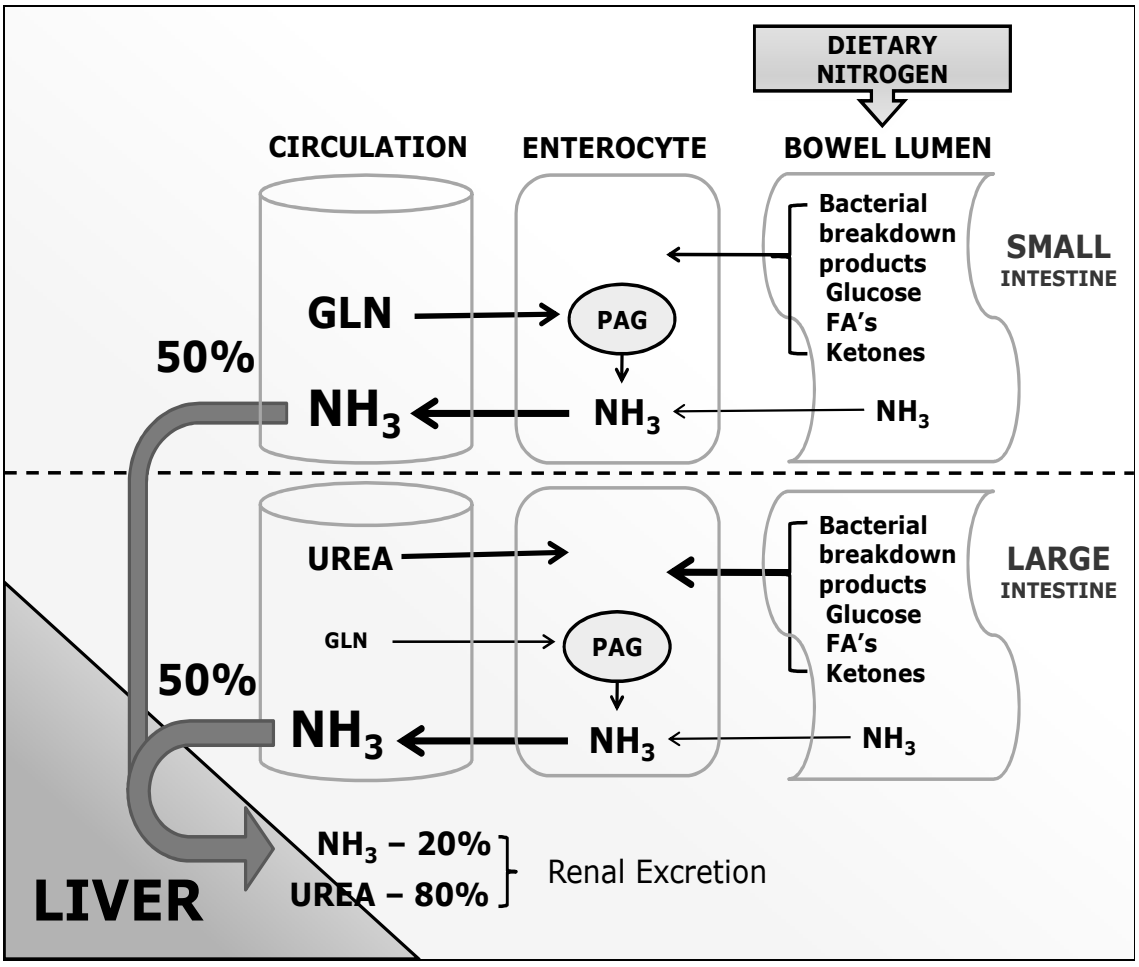
Lung: Ammonia lung tissue levels are in the milimolar range and about 5-10x circulating levels.(109) However, as suggested by studies in ALF pigs, there is a wide variation in lung ammonia metabolism probably due to differential regional arterial-venous and blood flow differences. However, in real terms the lungs contribute little to whole body ammonia.(109)

Heart: There is limited data on the heart's contribution to ammonia and glutamine metabolism with liver failure, but given the significant presence of GS(83) and PAG(96) the heart muscle could potentially have an impact.

Collectively the evidence for interorgan ammonia and amino acid metabolism in the post-absorptive state in both health and liver disease suggests that aside from the liver, systemic ammonia levels are chiefly determined by intestinal and renal ammonia metabolism, though with progressive liver disease skeletal muscle and the brain may have an increasingly significant roles.

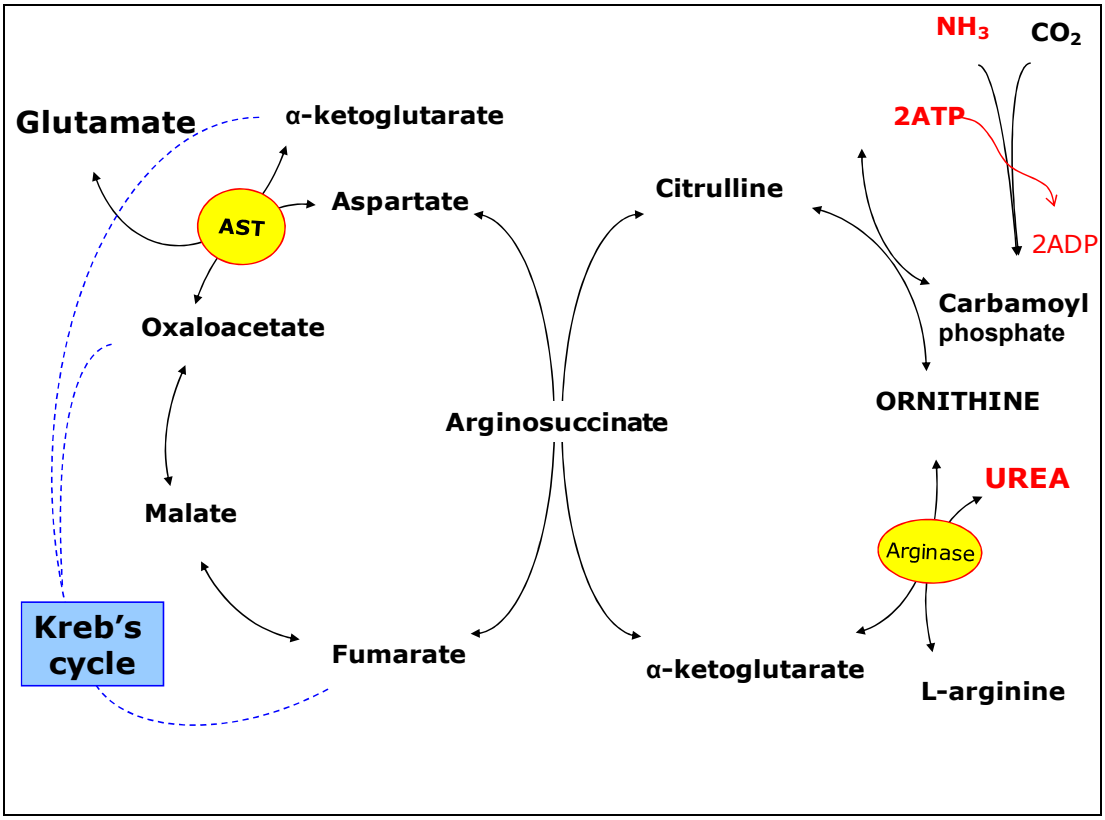
In conclusion, this chapter highlights the importance of interorgan ammonia and amino acid metabolism in health and diseased states with systemic ammonia predominately due to intestinal and renal ammonia efflux, with skeletal muscle providing a target for ammonia detoxification. This greater understanding may prove efficacious when developing future therapies to ameliorate the hyperammonemic state of liver disease.

Figure 1: Intestinal ammonia and amino acid production



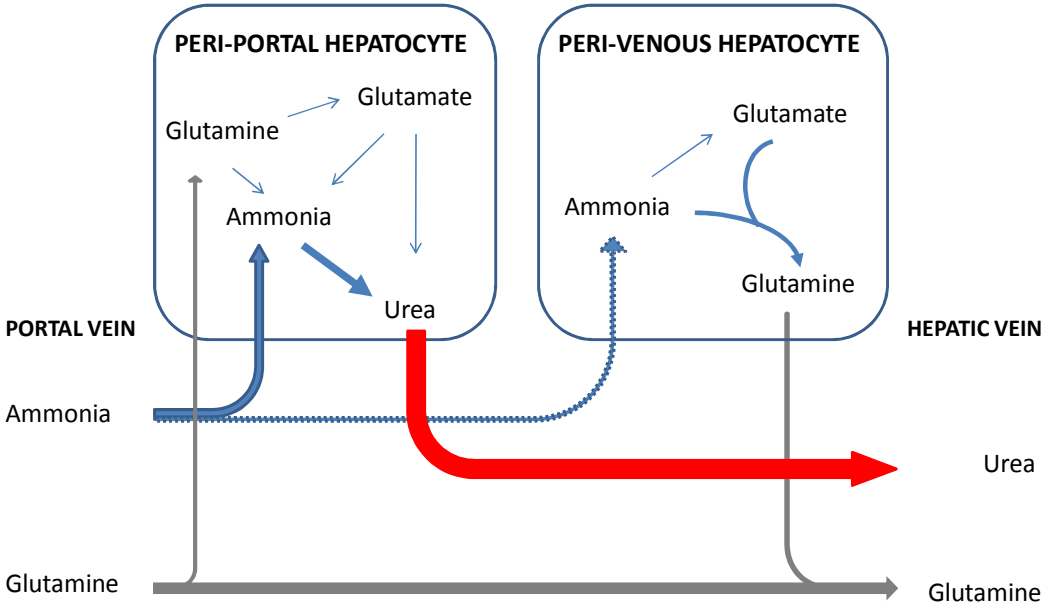
Schematic represents the relative contribution of circulating and dietary nitrogenous compounds in the small and large bowel to circulating ammonia. The small and large bowel contribute equally (50% each) of gut-derived ammonia. In the small bowel this ammonia is equally derived from luminal bacterial breakdown products (dietary nitrogen) and the rest from the conversion of circulating glutamine (from the superior mesenteric artery). In the large bowel only 8% is derived from circulating glutamine, 42% is derived from circulating alanine, but the majority is sourced from bacterial breakdown products in the lumen. Some 80% of gut-derived ammonia is converted to urea, the remaining 20% ends up as circulating ammonia.

Figure 2: Schematic of the interaction between the Urea and Kreb's cycle



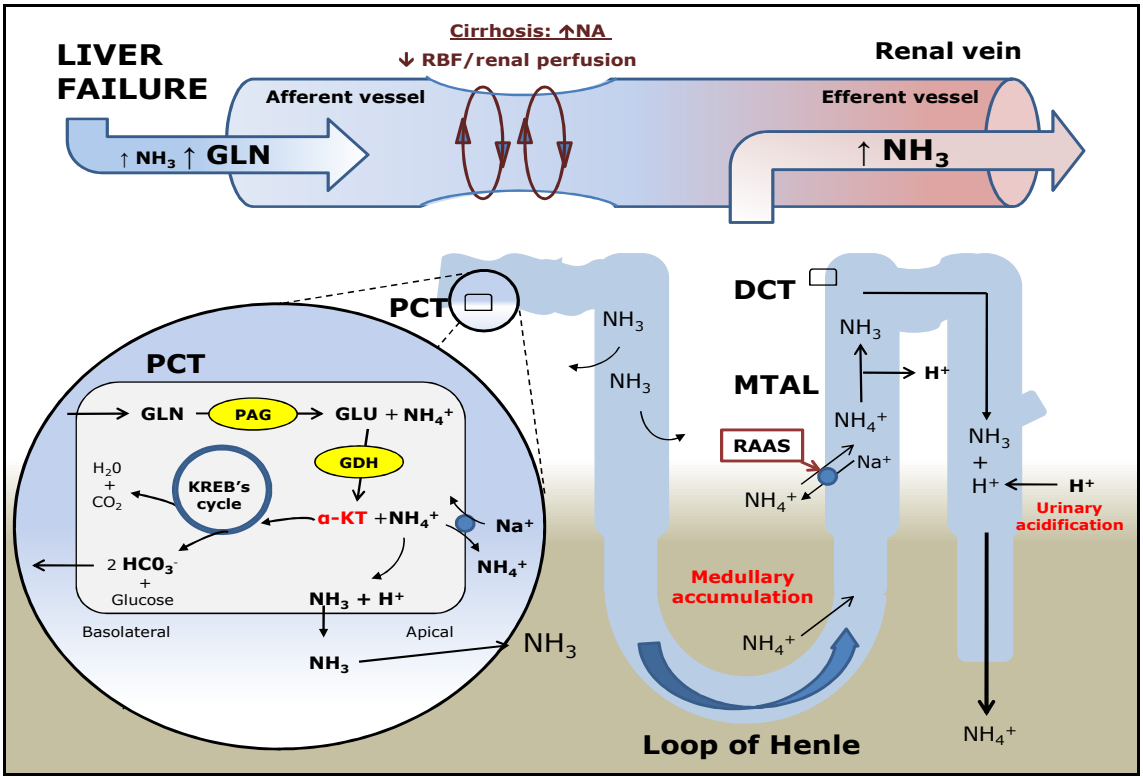
The urea cycle is closely linked to the Kreb's cycle deriving one of its nitrogens through transamination of oxalacetate to form asparate and returns fumarate to that cycle. This allows amino acids formed by the urea cycle to be used as a source of fuel in the Kreb's cycle; which is important as energy is expended through the urea cycle (~20% of the energy derived from metabolism of gluconeogenic amino acids).

Figure 3: Ammonia detoxification: glutamine cycling and ureagenesis



The compartmentalised hepatic urea and glutamine synthesis is very important in systemic ammonia detoxification, incorporating hepatic phosphate-activated glutaminase (PAG), acinar, and 'intracellular glutamine cycling' between periportal and perivenous hepatocytes. The highly abundant periportal hepatocytes contain the urea cycle culminating in the conversion of ammonia arriving from the portal vein to urea, the major end product of nitrogen metabolism. Periportal PAG converts any intestinal-derived glutamine into ammonia with a low-affinity and high capacity for its product (ammonia). PAG is also critical to the urea cycle, by providing intramitochondrial glutamate for N-acetylglutamate synthesis, activating CPT. Therefore, the bigger the supply of intestinal ammonia (and glutamine), the bigger its turnover to urea. Perivenous hepatocytes (7% of hepatocytes) surrounding terminal venules and are hugely abundant in glutamine synthetase (GS) (high affinity, but low capacity for ammonia) convert ammonia to glutamine. Therefore, if any ammonia escapes periportal hepatocytes, it can be scavenged and detoxified by perivenous hepatocytes to glutamine, which along with ammonia is passed into the hepatic vein.

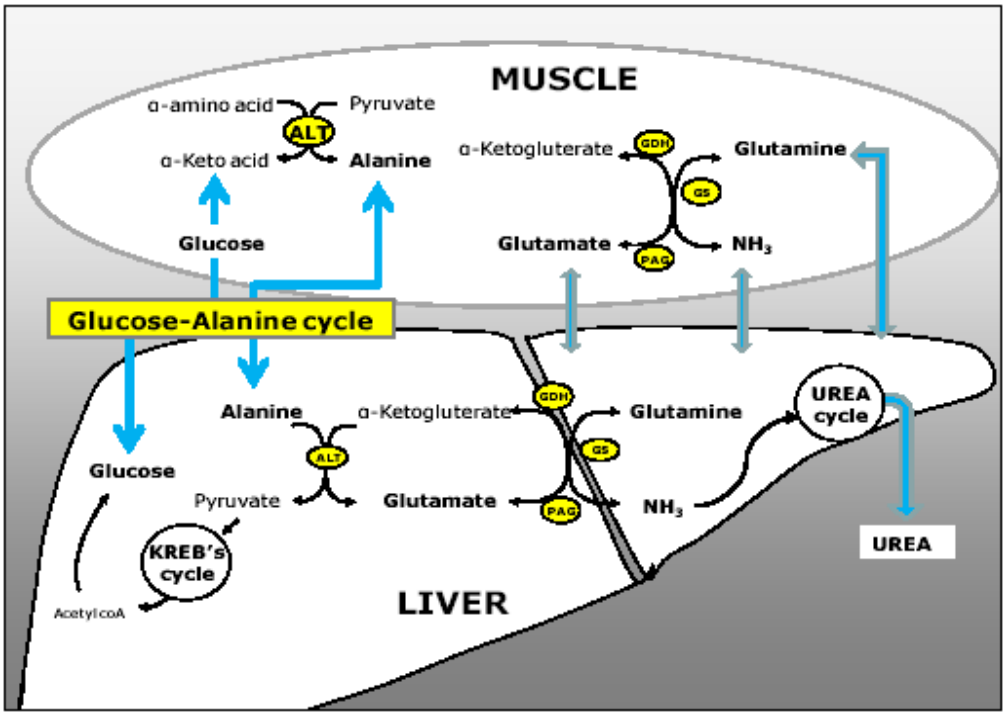
Figure 4: Renal ammonia and amino acid homeostasis



Glutamine (GLN) is the main substrate for renal ammoniagenesis. In the proximal collecting tubule (PCT), glutamine is converted to ammonia (NH_3) and glutamate (GLU) by phosphate-activated glutaminase (PAG), with renal PAG strongly inhibited by glutamate (not ammonia). Glutamate dehydrogenase (GDH) converts GLU to ammonia and α-ketoglutarate (α-KG) (energy substrate for Kreb's cycle). Ammonia (NH_3) is excreted into tubular fluid by non-ionic diffusion (along the pH gradient) and ionic diffusion after conversion to ammonium (NH_4^+) along electrical gradients via co-transporters (e.g. Na^+-NH_4^+). Conversion of NH_4^+ back to NH_3 and the countercurrent multiplier system sees NH_3 accumulate in the medullary interstitium of the ascending limb of the loop of Henle. This is quickly reabsorbed by the distal collecting tubules (DCT) of the medullary thick ascending limb (MTAL) as NH_4^+ via the Na^+-K^+- NH_4^+-$2Cl^-$ co-transporter (blocked by loop diuretics) allowing for urinary acidification and necessary buffering effect. Activation of the Renin-angiotension-aldosterone system (RAAS) dose dependently influences renal ammoniagenesis by directly increasing MTAL absorption via effects on co-transporters. In health, 70% of renal ammonia is released into the circulation (renal vein) with only 30% excreted in urine. However, with liver failure despite an initial increase in renal excretion with mild hyperammonaemia (renal adaption), triggered by increased muscle

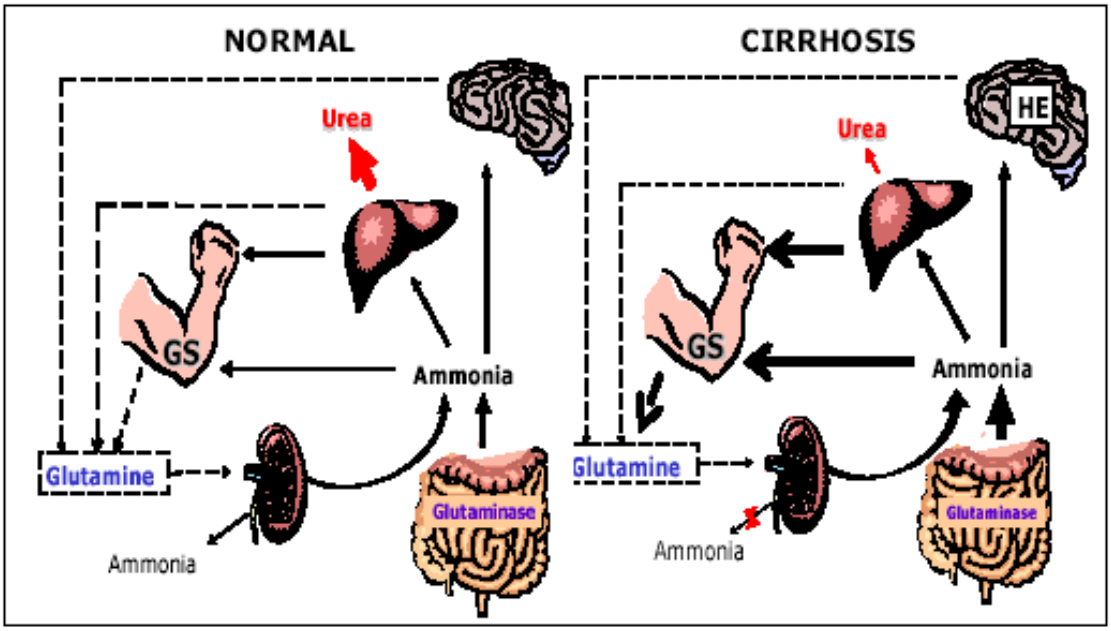
glutamine release and renal uptake (shifting GS and glutaminase activity), this is eventually overrun; as the kidney becomes a net ammonia producer given significant falls in glutamine uptake and ammonia excretion. Autoregulation maintains renal blood flow (RBF) and glomerular filtration over a wide range of blood pressures, but increased noradrenaline (NA) with renal syndromes of liver failure (e.g. HRS), reduces RBF and perfusion. Compromised renal function e.g. HRS) further reduces ammonia excretion. Metabolic acidosis also leads to net ammonia production (compensatory), by stimulating PCT GLN uptake and increased α-KG (and NH_3) with net circulating bicarbonate (HCO_3^-) synthesis. Therefore more NH_3 is excreted as HCO_3^- is conserved to compensate for the acidosis.

Figure 5: Skeletal muscle ammonia and amino acid metabolism



The interaction between key enzymatic processes in both skeletal muscle and liver have a profound influence on whole body ammonia and amino acid metabolism. Energy utilisation and increasing production of waste nitrogenous compounds such as ammonia, drives increased muscle glutamine synthetase (GS) activity and conversion to non-toxic glutamine which can be released into the general circulation, taken up by the liver and converted to urea (for excretion). The effect of phosphate-activated glutaminase (PAG) which catalyses the reciprocal reaction is less significant as it is markedly less abundant than GS. Additionally muscle pyruvate is transaminated to alanine by ALT and then transported to the liver. Once in the liver, alanine (with α-ketoglutarate) is converted to pyruvate and glutamine (also by ALT). The generated liver pyruvate can be incorporated into the Kreb's cycle by pyruvate dehydrogenase (PDH), oxidatively decarboxylated to acetyl coA and thus used as a substrate for gluconeogenesis which can be recycled back to muscle. - The 'glucose-alanine cycle'. The glutamate produced by this reaction also allows for the formation of ammonia (and glutamine) by liver PAG and later urea; with α-ketoglutarate recycled from glutamine dehydrogenase (GDH). The 'glucose-alanine cycle' therefore allows skeletal muscle to eliminate nitrogen while replenishing its energy supply. Therefore with the significant catabolism of fulminant liver failure low circulating glucose and high alanine levels, increases the relative importance of this pathway.

Figure 6: Interorgan ammonia metabolism in health and cirrhosis



In healthy individuals, the liver removes ammonia by detoxification into urea. In patients with cirrhosis, the metabolic capacity of liver is reduced, resulting in hyperammonaemia. The muscle then becomes an important organ for ammonia detoxification to glutamine. Glutamine acts as a temporary buffer that can both regenerate ammonia in the enterocyte and excrete ammonia via the kidneys.

Chapter 1.3

Hepatic Encephalopathy:

The Role of Inflammation

Introduction

Inflammation is the generalised response to injury and has come under increasing scrutiny as a precipitating factor in hepatic encephalopathy (HE). The 'systemic inflammatory response syndrome' (SIRS) refers to the clinical manifestation of inflammation (Table 1),(167) describing the consequences of dysregulated host responses following activation of a normally quiescent system, involving leucocytes, endothelial cells and cytokine networks. The clinical signs observed include body temperature, heart, tachypnea or, white blood cell count. Fever and leukocytosis are features of the acute-phase reaction, while tachycardia is often the initial sign of haemodynamic compromise. Tachypnea may be related to the increased metabolic stress due to infection and inflammation, but may also be an ominous sign of inadequate perfusion resulting in the onset of anaerobic cellular metabolism. Two or more of these criteria are necessary to diagnose SIRS, with it principally arising from overt infective sources. Although SIRS criteria are generally non-specific, they allow for an objective classification of critically ill patients, at least in the research setting. In advanced liver disease there is an increased susceptibility to both bacterial and fungal infections,(28) with more than 80% of patients with acute liver failure (ALF) having coexistent infection(25) with increased mortality. In liver failure, significant SIRS is associated with elevated circulating proinflammatory cytokines(27) which are independently associated with progression of HE.(29, 168, 169) SIRS is therefore closely linked to progression to advanced HE(26, 28, 169, 170) and independently - intracranial pressure (ICP),(169) likely by inflammation modulating the susceptibility of the brain to the effects of hyperammonaemia. It is also worth highlighting that SIRS can also originate from non-infectious (aseptic) sources, like drug-induced ALF, or ischaemic insult. This chapter discusses the current evidence supporting a role for inflammation in the development of HE.

Evidence for a role of inflammation in liver dysfunction

In the study by Rolando & colleagues,(28) from 887 ALF patients admitted over an 11-year period, the presence of SIRS components (Table 1) were recorded on admission and during episodes of infection. Fifty-seven percent of patients manifested a SIRS during their illness. There was a maximum of 1, 2, and 3 concurrent SIRS components in 19%, 27% and 11% of patients respectively. In the 39.8% of patients who did not become infected, the presence of a SIRS on admission was associated with worsening severity of encephalopathy and death. Not unsurprisingly, those patients with proven infection more often developed a more significant SIRS. In the 31% of patients with bacterial infection, the extent of the SIRS correlated with mortality. In those with a SIRS of 0, 1, 2, or 3, the mortality was 16.7%, 28.4%, 41.2%, and 64.7% respectively. Similar correlations between SIRS and mortality were seen with fungal infection, bacteraemia, and bacterial chest infection. In patients with severe sepsis or septic shock, 59% and 98% died respectively; demonstrating the close association between infection, SIRS and progressive HE. However, it was interesting to note that for patients with a SIRS manifesting worsening encephalopathy, the proportions of those who were infected compared to the non-infected patients were similar. These results tend to implicate proinflammatory mediators (e.g. cytokines and chemokines) as the key factors in the inflammatory response, rather than the microbial pathogen itself. So these findings suggest that in ALF, a SIRS independent of underlying infection is involved in the progression of encephalopathy and worsening prognosis.

We have previously reported a patient listed for transplant following paracetamol-induced ALF, who underwent a hepatectomy for critical hepatic necrosis after developing severe uncontrolled intracranial hypertension.(29) Hepatectomy resulted in stabilisation of both systemic and cerebral haemodynamics. The patient remained

anhepatic for 14 hours and was successfully bridged to orthotopic liver transplantation (OLT). Hepatectomy was associated with a sharp and sustained reduction in circulating proinflammatory cytokines, again suggesting that liver derived proinflammatory cytokines may be important in the pathogenesis of intracranial hypertension in patients with ALF. Elevated cerebral blood flow (CBF) fell from 73ml/100g/min pre-hepatectomy, to 43ml/100g/min 4 hours post-hepatectomy (normal range: 40-55 ml/100g/min), and remained within normal limits post transplantation. In this patient the haemodynamic changes noted were independent of arterial ammonia, which remained essentially stable (189-210μmol/l) pre- and post-hepatectomy, though fell post transplantation (89μmol/l). This observation pointed to a link between increasing CBF and ICP.

Further to these observations, Vaquero and colleagues,(26) as part of the U.S. acute liver failure study, undertook a prospective study of 227 consecutive patients with stage I-II HE, with the aim of identifying the predictive factors of worsening HE and the relationship with a SIRS and/or infection. At admission variables were analysed in acetaminophen (paracetamol) (n=96) and non-acetaminophen (n=131) ALF. In the acetaminophen group (on multivariate analysis) predictive factors of worsening HE were acquisition of infection ($p<0.01$) and increased leukocyte levels at admission ($p<0.01$). By contrast in non-acetaminophen patients, only increased pulse rate ($p<0.05$) and AST levels ($p<0.05$) at admission were predictors. In patients who progressed to deep HE, the first confirmed infection preceded progression in 79% of acetaminophen patients compared with 52% of non-acetaminophen patients. In patients with negative microbiologic cultures, a worsening SIRS (0, 1, and ≥2) at admission was associated with severity of HE, 25%, 35% and 50% respectively ($P<0.05$). This again shows the importance of infection and/or the resulting systemic inflammatory response in the progression of

HE in ALF. It is worth noting that current laboratory techniques for confirming endotoxaemia/infection are imprecise, such that high SIRS could reflect underlying infection even if not detected.

Nature of liver injury

There is interest in what possible interplay exists between inflammation, chronicity of liver disease and presentation of HE. Research has often focused on ALF, due to the defining encephalopathy that ensues. Increased ICP and cerebral oedema, though typical of ALF, is rare in patients with cirrhosis.(2, 3) However, there are case reports of similar effects with acute liver injury superimposed on a background of cirrhosis, termed 'acute-on-chronic liver disease' (ACLF), defining a group of patients with a chronic 'phenotype' who appear clinically indistinct from those with ALF. In these patients the trigger for acute deterioration is often the presence of a precipitating event such as sepsis, or an increased ammonia load (secondary to gastrointestinal bleeding or the creation of a portosystemic shunt). One such precipitant in cirrhotic patients is 'transjugular intrahepatic portosystemic shunts' (TIPS), used to manage uncontrolled variceal haemorrhage, or diuretic resistant ascites. In study of 56 patients who underwent emergency TIPS for uncontrolled variceal haemorrhage, 4 developed features of ACLF, with marked deterioration in liver function tests (LFTs) and elevated ICP.(32) From subgroup analysis there were no clear causal factors making patients more susceptible to ACLF following insertion of a TIPS. Although there was a trend to lower plasma sodium in the patients who developed intracranial hypertension post-TIPS, this was not statistically significant. However, out of the 4 who developed ACLF, 1 had developed aspiration pneumonia prior to developing a raised ICP and cerebral oedema. Also, another 2 of the 4 cases had evidence of cerebral ischaemia post-mortem. Both infection and

ischaemia are associated with a significant proinflammatory cytokine response, hinting at a role for inflammation in the progression of encephalopathy in ALF.

The role of inflammation in acute liver failure

As already discussed, studies in patients with ALF have shown the rapid progression to severe HE in those with evidence of a SIRS, suggesting a possible link between inflammation and HE(26, 28) especially in those with more advanced disease (rising ICP and cerebral oedema). Though hyperammonaemia has been widely implicated, the mechanism for these changes remains unknown.

Inflammation - Effects on cerebral blood flow in ALF: There is growing evidence that disruption to CBF autoregulation may underlie the pathogenesis of increased ICP in patients with ALF.(171) This is potentially through alterations in the inflammatory milieu; addressed in a section of this chapter called – *'Multicellular inflammatory interactions and cerebral haemodynamics'*. A recent study was undertaken to determine the role of inflammation in the pathogenesis of ICP in patients with ALF and its interplay with CBF and ammonia.(169) Twenty-one patients with ALF were studied from the time they were ventilated for severe encephalopathy, until receiving specific treatment for increased ICP. Depending upon the ICP, the patients were divided into two groups - group 1 (n=8) requiring specific treatment for ICP>20 mmHg (mean ICP=32 mmHg (range 28-54) and group 2 (n=13) those with ICP≤20mmHg (mean ICP=15 (range 10-20) mmHg) not requiring treatment. In the group 1 patients, inflammatory markers, arterial ammonia and CBF were significantly higher. Significantly, both tumour necrosis factor-alpha (TNF-α) levels, and ICP directly correlated with CBF (r^2 = 0.80 and r^2 = 0.74 respectively). Interestingly, in 4 patients from group 2 who developed isolated

surges of increased ICP, there were associated increases in markers of inflammation, TNF-α and CBF. These results support some of our earlier findings and support the hypothesis that inflammation plays an important synergistic role in the pathogenesis of increased ICP, possibly through its effects on CBF. However, one should understand that brain ammonia may remain stable with worsening disease as ammonia delivery reduces with falling CBF.

Hypothermia - Modulation of inflammation and haemodynamics in ALF: 20% of patients with ALF die from increased ICP while awaiting transplantation. However, there has been anecdotal evidence for the beneficial role of hypothermia treatment for intracranial hypertension in neuropathological disorders and also more recently ALF. Given the established role of cytokines in pyrexia (i.e. interleukin-1 (IL-1)) and ALF, it has been hypothesised that the beneficial effects of hypothermia are enacted by cytokine responses. The clinical effects and pathophysiological basis of hypothermia was studied in 14 ALF patients awaiting OLT who had increased ICP unresponsive to standard medical therapy.(156) Core temperature was reduced to 32-33°C using cooling blankets. Thirteen patients were successfully bridged to OLT with a median of 32 hours (range, 10-118 hours) of cooling. They underwent OLT with no significant complications related to cooling either before or after OLT and had complete neurological recovery. ICP was significantly reduced ($p<0.0001$) from 36.5 ± 2.7mmHg before cooling to 16.3 ± 0.7mmHg at 4 hours, which was sustained at 24 hours (16.8 ± 1.5mmHg). Mean arterial pressure (MAP) and cerebral perfusion pressure (CPP) increased significantly, and the requirement for inotropes was reduced significantly. Hypothermia was seen to produce a sustained and significant reduction in CBF, brain cytokine production ($p=0.01$), markers of oxidative stress, arterial ammonia concentration and also ammonia brain

metabolism. This study shows that hypothermia reduces ICP by impacting on cytokine responses and other pathophysiological mechanisms and acts an effective and safe bridge to OLT.

The role of inflammation in cirrhosis

The pathophysiological basis of ACLF is just as unclear, but the systemic inflammation is equally thought to be important. On the background of a correlation between SIRS and progression of HE in ALF, the hypothesis that SIRS mediators such as nitric oxide (NO) and proinflammatory cytokines may exacerbate the neuropsychological effects of hyperammonaemia in well compensated cirrhosis was tested.(170) Hyperammonaemia was induced (with an oral amino acid solution to simulate an upper G.I bleed) in 10 patients with cirrhosis 24-36 hours after admission with clinical evidence of infection. They were followed during a period of treatment with conservative management (including antibiotics) and after resolution of the infection. The hyperammonaemia generated in response to the amino-acid solution was similar prior to, and after resolution of the inflammation/infection. With treatment of the infection there were significant reductions in the white cell count (WCC), C-reactive protein (CRP), nitrate/nitrite ($p=0.002$), and the proinflammatory cytokines - IL-6, IL-1β and TNF-α (0.002, 0.03 and 0.03 respectively). An earlier study demonstrated that the hyperammonaemia induced by a simulated bleed alone has no significant neuropsychological deterioration in stable cirrhotics.(172) In this study, in those patients with a SIRS, the induced hyperammonaemia resulted in a significant worsening of neuropsychological scores that was not seen in patients without a SIRS. Significantly, the neuropsychological effect resolved following resolution of the infection and SIRS. On the background of hyperammonaemia, such inflammatory related neurological changes with the

associated reduction in cytokines and nitric oxide, suggest that inflammation and its mediators may be important in modulating the cerebral effect of ammonia in cirrhosis and ACLF.

***Inflammation - Role in minimal hepatic encephalopathy**:* It is now recognised that minimal hepatic encephalopathy (MHE) is common in patients with cirrhosis. In cirrhotic patients who are well post transplantation, reversal of abnormalities in neuropsychological function may not always be complete(173) as is often believed. This observation, points to factors other than ammonia in the pathogenesis of less severe encephalopathy. The extent to which precipitants of HE such as infection and ammonia interact with each other to cause different grades of encephalopathy is still little understood. A further study was set up to determine whether the presence of MHE was associated with ammonia levels, inflammation and severity of liver disease.(174) Eightyfour patients with cirrhosis were put through a neuropsychological test battery and had serum samples taken. These parameters were tested prior to, and 4 hours after induction of hyperammonaemia by oral administration of an amino acid (n=60) or a placebo solution (n=24). Administration of the amino acid solution did induce deterioration of some neuropsychological function tests, but none developed overt HE. In those who received placebo there was no deterioration in neuropsychological function. The immediate recall was the most affected test, with 55% (33/60) deteriorating from baseline, 43% (26/60) deteriorated from baseline with 'trails B test' (although 16 had abnormal baselines), 18.3% (11/60) with 'digit symbol substitution test' and 15% (9/60) with 'choice reaction time'. However, this neuropsychological deterioration following induced hyperammonaemia was independent of the magnitude of change in plasma ammonia and severity of liver disease, yet

significantly greater in those with evidence of inflammation. These data indicate a role for ammonia in HE, but suggest that the presence and severity of MHE is independent of the actual plasma ammonia concentration and stage of liver disease. Significantly, inflammation again would appear to play an important role in determining the baseline neuropsychological state and also in modulating the neuropsychological effects of ammonia.

Inflammation: role of the molecular adsorbents recirculating system: Recently, there has been a lot of interest in liver support systems as a therapy for liver failure. In patients with ACLF, 'the molecular adsorbents recirculating system' (MARS) improves individual organ function. But the mechanism by which it exerts an effect in the treatment of HE is still unclear. Given data showing the likely importance of mediators of inflammation in HE, a study was performed to determine if the therapeutic effect of MARS similarly involved inflammatory mediators and/or ammonia.(175) Eighteen patients with acutely decompensated alcohol-related cirrhosis precipitated by inflammation were randomised to receive either standard medical therapy (SMT) alone, or SMT with MARS therapy over 7 days and inflammatory mediators and ammonia measured. Compared to SMT, encephalopathy improved significantly with MARS ($p<0.01$). However, we did not observe any significant change in plasma cytokines and ammonia levels in either group, nor did malondialdehyde (MDA), an indicator of oxidative stress. In fact the only indicator of inflammation that correlated with the observed clinical improvement with MARS therapy was the nitrate/nitrite ratio (NOx), $p<0.05$. The improvement seen with MARS could not be explained by haemodynamic changes either, as both MAP and renal function remained unchanged. It therefore appears that in inflammation-related ACLF, albumin dialysis using MARS results in

improvement of encephalopathy, independent of changes of ammonia, cytokines, or improved haemodynamics; possibly via a NO-dependent pathway.

The role of the blood brain barrier

Ordinarily the blood brain barrier remains intact during liver disease(176, 177) and should theoretically prevent the entry of cytokines from the systemic circulation into the brain. However, data from other neuropathological inflammatory states show that proinflammatory cytokines (e.g. TNF-α and IL-1) may produce brain swelling by inducing an increase in the permeability of the blood brain barrier (e.g. bacterial meningitis(178, 179)). Evidence from cell-culture studies supports TNF-α modulation of barrier permeability.(180) In one such study intracisternal administration of TNF-α caused a dose-dependent increase in barrier permeability,(179) while systemic administration of TNF-α appears less effective in disrupting the barrier.(181, 182) Despite an earlier animal study of liver failure describing some degree of barrier disruption,(183) this has not been a consistent finding. Such inconsistencies in the barrier integrity of animal models of liver failure may be due to differences in HE severity, with the more advanced brain sequelae associated with a loss of barrier integrity. Support for barrier breakdown during the late stages of disease has recently been provided in a recent communication demonstrating a loss of blood brain barrier integrity with severe sepsis.(184) In rats that were made septic using caecal ligation and puncture, brain electron-microscopy showed severely swollen astrocytes with evidence of breakdown of the tight-junctions with entry of lanthanum into the brain.(184)

There is a direct correlation between HE grade and the presence of a systemic inflammatory response. However, given that cytokines are 15–20 kD in size and cannot directly cross an intact blood–brain barrier, it was thought that they may not

have a direct effect on brain microvessel endothelial cells.(185) However other studies suggest alternative routes of activation of the brain by peripheral cytokines.(186) Researchers have shown direct brain signalling to the brain via the vagus nerve by the peripheral and autonomic nervous systems.(187) In the periphery, inflammatory mediators acting on vagal afferents will activate the nucleus tractus solitarius, the primary projection of the vagus nerve(188) and subdiaphragmatic vagotomy will abrogate the induction of IL-1β messenger RNA in rat brain by peripheral IL-1β.(189) It is possible that cytokines cross the blood brain barrier by active transport or enter the brain at areas that lack a barrier. However, evidence supports a role for secondary messengers in response to cytokines binding to their receptors expressed on brain microvessel endothelial cells,(190) e.g. TNF-α, IL-1β and bacterial lipopolysaccharide (LPS) activation of cGMP-dependent protein kinases leading to inducible NO synthase (iNOS) production.(191, 192) Without activation by microbial pathogens or proinflammatory cytokines, free-radicals and oxidative stress are likely to be just as crucial in the pathogenesis of ammonia-induced neurotoxicity. In hyperammonaemia, free-radical production may be mediated by NMDA-receptor activation and ammonia-induced mitochondrial dysfunction.(193) This could also be a source of reactive oxygen species such as peroxynitrite (OONO•), which mediates NO-induced blood brain barrier damage. Furthermore, antioxidants have beneficial effects in experimental animal models of HE,(155) and hyperammonaemia.(194)

Local cerebral inflammatory response in progression of encephalopathy

Given all the presented data on the systemic inflammatory response, it is important to discuss what local triggers may enact this circulatory response. The most obvious organ-specific inflammatory insult involved in HE would clearly be the hepato-

cerebral axis. However, the extent of liver inflammation does not directly correlate with HE grade. With an intact barrier during progressive HE, theoretically the brain should not mount a direct response to systemic derived cytokines as part of SIRS. However, certain cell types within the brain may self-propagate a cytokine response (discussed later). A study was therefore undertaken to assess if an exaggerated brain net production (flux) of brain cytokines in response to acute liver injury exists as a possible trigger for HE.(195) The 'flux' of an organ (e.g. jugulovenous–arterial x CBF) is a better determinant of organ metabolism than the arterial-venous concentration gradient, as it eliminates blood flow across the organ as a confounding variable. Sixteen mechanically ventilated ALF patients were studied with the measurement of CBF (Kety-Schmidt technique) and ICP (Camino subdural catheter).(195) Study groups were divided by differing ICP into 1) normal, 2) controlled (>20mmhg), or 3) uncontrolled ICP (>25 mmHg) despite treatment (mannitol and/or haemofiltration). Blood was sampled from an artery and the reverse jugular catheter to measure proinflammatory cytokines and ammonia. Plasma ammonia between groups was not statistically significant. However, there was a good correlation between arterial proinflammatory cytokines and ICP (r^2 = 0.34, 0.50 and 0.52; for IL-6, IL-1β and TNF-α respectively).(195) There was also a positive cerebral cytokine flux in ALF patients with uncontrolled ICP.(195) Additionally, in 3 patients studied longitudinally (serial sampling) over 72 hours, brain proinflammatory cytokine production was only seen with uncontrolled ICP (Figure 1).(195) These results indicate significant activation of brain proinflammatory cytokines in patients with advanced HE and a possible compromised blood brain barrier at this late stage (given the appearance of these cytokines in the jugular bulb catheter). Such results allude to a critical role of brain-derived proinflammatory cytokines in the transition of a patient to the advanced

stage of intracranial hypertension where they may become refractory to standard medical intervention. This cerebral cytokine flux may be the critical trigger for CBF modulation, increased blood brain barrier permeability and intracranial hypertension. However, it is equally probable this cerebral cytokine flux is the response to an already activated systemic trigger such as cytokines, which then critically modulate cerebral inflammatory response. Earlier studies suggest the systemic inflammatory state of ALF patients may represent an effect of progressive liver necrosis and/or associated infection. Modulation of hepatic cytokine release may be the pathophysiological basis for the benefit of hepatectomy in ALF shown in previous studies.(29, 196, 197) All these studies support the paradigm of a likely systemic cytokine driven inflammatory cascade as the important determinant of cerebral effects in acute liver injury, modulating or modified by any local brain inflammatory response).

Multicellular inflammatory interactions and cerebral haemodynamics

The presented studies do not allow one to draw any conclusions as to which cell types in the brain are involved in inflammatory responses (as demonstrated by proinflammatory cytokine production), but a number of cell lines in the neurovascular unit are capable of cytokine responses such as glial cells (astrocytes and microglia), pericytes, brain microvascular endothelial cells as well as migrating cells from the circulation. Astrocytes are thought to be the cells most likely to be involved because they are known to be central to blood brain barrier integrity, have an extensive cytokine repertoire(198) and swell in ALF. As cytokines are able to modulate glial cell activation this finding could explain the effects of cytokines at the barrier and resultant effect on cerebral haemodynamics seen in ALF. This would suggest that the transition of patients with ALF to uncontrolled ICP may indicate a

critical neuropathological change involving modulation of cerebral haemodynamics triggered by proinflammatory cytokine responses.(169) This hypothesis will have to be tested in appropriate studies.

Cerebral autoregulation is an important concept,(199) which describes the maintenance of a constant CBF over wide MAP, with buffering of CPP ([CPP = MAP – ICP] with a normal range of 60-160mmHg) by dilatation or constriction of cerebral resistance vessels. In ALF, there is dysregulated cerebral autoregulation, with rising ICP directly correlated with ICP.(169) In contrast with the hyperaemia seen in ALF, cirrhosis is associated with cerebral oligaemia, except in a few cases where a second hit leads to ACLF, which has a phenotype similar to ALF. It remains unclear how much myogenic (smooth muscle), neurogenic, metabolic (energy demands) or multiple brain cell types (e.g. smooth muscle cells, neuronal inputs, astrocytes, pericytes and endothelial cells) interact to trigger changes in cerebral haemodynamics. The smooth muscle of arterioles contracts more as stretching increases, activating phospholipase C and arachidonic acid (AA) pathways (and derivatives) to orchestrate changes in potassium-dependent calcium channels and thus action potentials along the muscle. In regards to neuronal innervation, noradrenaline (NA) (locus coeruleus), serotonin (raphe nucleus) and dopamine (mesencephalic ventral tegmental area) nerve endings trigger constriction, while acetylcholine (Ach) (basal forebrain) and glutamatergic nerve endings cause dilation (along with elevation of intracellular calcium). Current reports highlight the effects of both neuronal activation and cyclooxygenase (COX)/AA and NO inflammatory pathways on astrocytes.(165, 200, 201) However, their findings (especially between Zonta et.al. and Mulligan et.al.) draw differing conclusions on the relative downstream effect on activation of this differing pathways on vascular tone; Mulligan et.al., demonstrating NA induced astrocyte AA production leading to

arteriolar constriction via 20 HETE, and Zonta et.al., pointing to NA induced astrocyte AA activation and COX-dependent PGE_2 induced vasoconstriction. In regards to energy demands, metabolically active neurons may lead to glutamate overspill, causing downstream activation of astrocytes with production of AA derivates such as epoxyeicosatrienoic acid (EET) a vasodilator, formed by cytochrome (CYP) P450. EET diffuses to nearby arteriolar smooth muscle to antagonise HETE (a vasoconstrictive AA derivative(201)) by activation of potassium-dependent calcium channels. But control of cerebral vascular tone is not as simple as the dynamic balance between AA products such as HETE (constrictor) and EET (dilator), as there are many other vasoactive metabolites. Astrocytes may release other vasoactive signals (e.g. thromboxane A2, prostaglandins (PgE and PgF) and prostacyclin). The potassium-dependent calcium channels, may also be affected by other mediators (e.g. NO, extracellular potassium, adenosine and prostacyclin). Neither should it be forgotten that glutaminergic neurons produce the powerful vasodilator NO, or the involvement of other brain cell types like microglia and endothelial cells (especially with their significant NO secondary messenger role linking systemic to brain inflammatory effects).

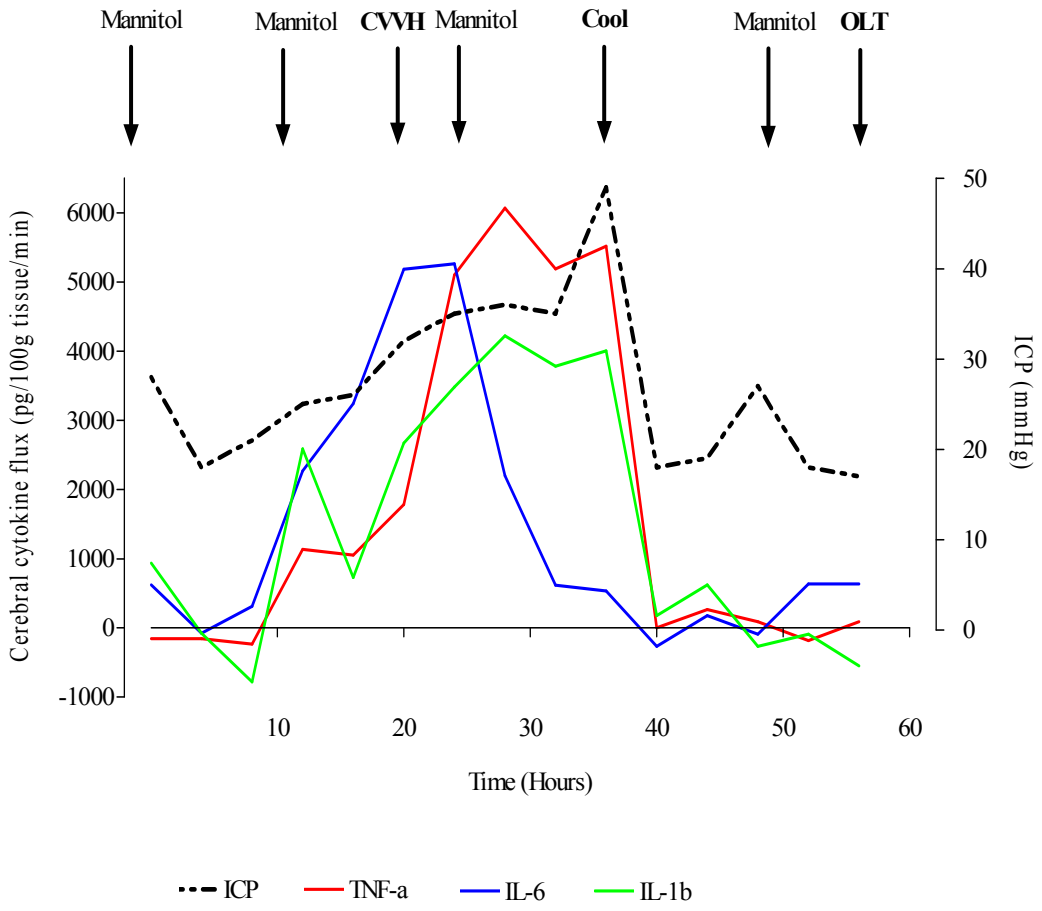
A recent study has shown a 2-3-fold increase in brain (as well as plasma and cerebrospinal fluid) mRNA expression of the proinflammatory cytokines (TNF-α, IL-1β and IL-6) at coma stages of encephalopathy with induction of ALF (hepatic devascularised rats) which correlated to HE severity and progression of brain oedema.(202) Microglial activation (indicated by upregulation of CD11b/c immunoreactivity) was also noted along with mild hypothermia delaying the onset of encephalopathy, prevented brain oedema and concomitantly attenuating brain ($p<0.001$), plasma and CSF proinflammatory cytokines.(202) This beneficial anti-inflammatory effect on microglia has also been recently demonstrated in a further

study utilising the anti-inflammatory agent, Minocycline.(203) These findings support much of the data presented above, and indicates that in ALF the brain can produce cytokines with evidence of actual production in those with uncontrolled increases in ICP. This study and other more recent work by the same group(203) begin to implicate microglia in the progression to HE on the background of evidence linking microglia in neuroinflammation (evident on positron emission tomography studies(204, 205)) influenced by systemic inflammatory responses.(206) Furthermore, recent studies suggest a critical bidirectional role for pericytes in cerebral haemodynamics.(207) Pericytes are mesenchymal-like, multipotent (relatively undifferentiated cells) with immune 'macrophage-like' antigen presenting properties. They also contractile and wrap around cerebral capillaries often enclosed within the basal lamina, especially at junctions, though less abundant in arterioles & venuoles. Interestingly, though capillaries lack smooth muscle, 65% of NA (purinergic) nerve terminals end here. These properties allow for pericytes to regulate cerebral blood flow downstream from arterioles in response to sympathetic neuronal signalling, with NA and ischaemia directly causing constriction, reversed by glutamate.(207) This suggests a possible hierarchy for cellular interactions regulating cerebral haemodynamics (e.g. smooth muscle cells, neuronal inputs, astrocytes, pericytes and endothelial cells). If one was to extrapolate these findings to the pathophysiology of HE, one could hypothesise that in conjunction with the role of astrocytes on cerebral haemodynamics,(165) pericytes may be the gatekeepers of microvessel tone (resistance vessels), accounting for both the oligaemia of cirrhosis (with possible chronic pericyte over-activation) and hyperaemia of ACLF and ALF (with pericyte inhibition). Given the reported effects of both neuronal activation and COX/AA and inflammatory pathways on both these cell types,(165, 200, 201, 207) one can begin to better understand the paradigm for HE

pathogenesis, with the fundamental roles of ammonia (via effects on astrocytes), inflammation and cerebral haemodynamics. However, whether it is the systemic inflammatory response that triggers brain inflammatory responses remains unclear.

In conclusion, this chapter outlines the existing data on the potential role of inflammation in progression of HE. The evidence presented supports the hypothesis that inflammatory responses are integral to the progression of hepatic encephalopathy via modulation of the cerebral affect of ammonia which impact on CBF autoregulation, ICP, barrier integrity and likely neuronal function. Further work will be necessary to elucidate the mechanisms of such inflammatory mediators at the neurovascular interface and cellular levels, but should lead to a better understanding of the role of inflammation in HE which may allow for the development of novel interventions targeting inflammatory pathways to treat HE.

Figure 1: Longitudinal cytokine fluxes following intervention for ALF



Represents the change over time of ICP and cerebral cytokine fluxes (TNF-α, IL-1β and IL-6) in a patient with ALF and raised ICP that is uncontrolled with standard medical therapy, patient 1. However, the application of hypothermia resulted in a rapid reduction in the production of cytokines from the brain to values not significantly different to zero [Adapted from a figure published by Wright G, Shawcross D, Olde Damink SW, Jalan R. Brain cytokine flux in acute liver failure and its relationship with intracranial hypertension. Metab Brain Dis 2007;22:375-388].

Table 1: SIRS components

SIRS components	
Respiratory	Rate ≥ 20 breaths/min, or PaCO2 ≤4.3 kPa
Cardiovascular	Heart rate ≥90 beats/min
Metabolic	Temperature ≥38° C (or ≤36° C)
Biochemical	White cell count ≥12 (or ≤4 x10^9/L), or ≥10% immature neutrophils

Each SIRS component scores 1; in presence of infection a score of ≥2 indicates sepsis [Adapted from the following publication - American College of Chest Physicians/Society of Critical Care Medicine Consensus Conference: definitions for sepsis and organ failure and guidelines for the use of innovative therapies in sepsis". Crit. Care Med. 20 (6): 864–74. 1992].

Chapter 1.4

Hepatic Encephalopathy:

Management of HE

Introduction

The wide spectrum of neuropsychiatric presentations of HE has lead to a plethora of therapeutic approaches. The type of treatment used is predominately dictated by the chronicity and severity of encephalopathy, with a need for more interventional therapies for those patients with acute liver injury advanced HE compared to those with MHE. Irrespective, reduction of plasma ammonia remains the central strategy, although randomised controlled trials point to the potential usefulness in particular circumstances of novel strategies like Flumazenil and Bromocriptine (see later). This chapter discusses the current evidence supporting the use of these principle therapeutic interventions for both the general management of chronic HE and that necessary for more acute and advanced disease.

GENERAL MANAGEMENT OF CHRONIC ENCEPHALOPATHY (Table 1)

Ammonia lowering strategies

Gastric lavage

Gastric lavage is only indicated where HE is precipitated in patients with cirrhosis by an acute upper gastrointestinal bleeding.

Dietary protein supplementation

Patients with cirrhosis (especially if alcohol-induced), usually have a poor nutritional reserve due to anorexia, poor diet, malabsorption, and altered metabolic state. Hospitalized patients are often hypermetabolic and hypercatabolic, worsened by complications such as gastrointestinal bleeding, continued anorexia and fasting for tests. The vogue for dietary protein restriction for patients with cirrhosis as a

plausible intervention for HE has recently been dispelled following a clinical trial showing that protein restriction does not improve HE, but may instead be potentially harmful.(208) In this study, the group treated with low-protein diet were shown to have increased protein catabolism. Current evidence suggests that protein requirements are increased in patients with cirrhosis. High protein diets are therefore well-tolerated in patients with cirrhosis,(41) with protein restriction but high calorie intake with inborn errors of metabolism the only exception. As such the consensus review by the 'European Society for Parenteral and Enteral Nutrition' (ESPEN) recommends a normal, or higher supply of dietary proteins (1-1.5 g/kg protein and 25-40 kcal/kg per day).(41, 42)

Branched-chain amino acids (BCAAs)

BCAAs are chiefly derived from dairy products and vegetables, make up 25% of total dietary protein content. They are a good substrate for protein synthesis, both conserving and restoring muscle mass in advanced liver disease. In cirrhosis, poor dietary intake leads to a deficiency of BCAA and resultant accumulation of aromatic amino acids. This creates a marked protein-energy deficit and increases false neurotransmitter precursors, altering glutaminergic neurotransmission. In the few 'high protein diet' intolerant and severely malnourished patients, BCAA supplements may be useful to provide the necessary nitrogen intake without a decline in mental state. As BCAA are under the influence of circulating insulin, the insulin resistance state of cirrhosis, limits their nutritional benefit unless systemic insulin replacement is implemented. Also, enteral nutrition (whether oral or nasogastric) is preferential to parenteral nutrition as prolonged TPN may induce fatty liver and inflammation especially in patients with short-bowel syndrome and also increases the risk of infection. However, a number of meta-analyses have failed to find consensus on the

use of BCAA in cirrhosis from a wealth of conflicting data.(209, 210) In most cirrhotic patients a modified eating pattern, based on several meals and a late evening snack, usually provides an adequate nutritional intake.(209, 211)

Glycaemic control

Disturbed glycaemic and lipid control is common in progressive liver disease and worsened by the stress response in critically unwell patients. Therefore in the hospitalized patient, once feeding has commenced, tight glycaemic control using insulin may be necessary to 1) reduce oxidative stress (which triggers insulin resistance), limit mitochondrial liver damage, and improve endothelial activation (e.g. NO production), which will improve blood flow, limiting tissue injury, and improve outcome.(212, 213)

Vitamins and nutrients

Cirrhosis also leads to deficiencies of lipid-soluble vitamins, minerals and micronutrients. For example, Zinc is a cofactor in the urea cycle(214) and also found in vesicles of predominately glutamatergic presynaptic terminals thereby having a role in neurotransmission.(215) Zinc supplementation (600mg/day) has been studied without obvious benefit, though replacement should be considered if the patient is deficient.(216) Manganese has also been implicated, as autopsy specimens from patients with hepatic coma and pallidal MR images of patients with HE suggest manganese deposition in the basal ganglia is involved.(11, 217) However, as with earlier studies evaluating the role of gut bacterial products like Mercaptans, Phenols and medium- and short-chain fatty acids,(218) there has been little cumulative evidence to support targeted treatment strategies to influence the management of HE.

Prebiotics, Probiotics and Synbiotics

Ammonia produced by the gut is derived impart from the deamination of dietary amino acids by bacteria, with a small contribution from the urea produced by urease-positive bacteria. In the critically ill and malnourished patient, levels of the predominant defensive bacteria strains (*Bifidobacterium spp.* and *Lactobacillus spp.*) decline. Antibiotics may further lead to ammonia-producing bacteria ameliorating hyperammonaemia. Biotics refer to "of, relating to, or caused by living organisms." In the digestive system, Prebiotics (non-digestible food compounds that stimulate the growth and/or activity of other beneficial bacteria (Probiotics)), and synbiotics (symbiosis of pro- and prebiotics), contain living/non-living non-pathogenic micro-organisms utilized as food ingredients, may have a role in the treatment of HE. They are thought to exert an effect in HE by 1) reducing intestinal ammonia production by enterocyte glutaminase, 2) reduce bacterial translocation, 3) modulate proinflammatory responses, and 4) modulate gut permeability.(219) Furthermore, probiotics bypass the small bowel and get fermented by colonic bacteria to form lactic, acetic and butyric acids, and gas (mainly hydrogen); any resultant intestinal hurry may increase the expulsion of ammoniagenic bacteria. In randomised placebo controlled trials,(220) probiotics have been shown to reduce gut ammonia production and inflammation(220, 221) with synbiotics leading to an improvement in MHE (associated with an increase in faecal pH, reduced plasma endotoxin and ammonia levels).(220, 221) It is worth noting that fermentable fibres alone were also beneficial in that study. This is not unexpected as the common effect of probiotics, as with prebiotics, aside from a decline of substrate for other bacteria(222) and reduction on bacterial translocation, is the fermentation of non-absorbed sugars (e.g. mono-, di- & oligosaccharides). This fermentation of sugars

leads to the production of differential amounts of lactic acid, ethanol and CO_2 to modulate intestinal acidity and gas production.

Purgatives

A purgative is an agent, which cleanses the bowel by increasing the evacuation of luminal contents. This is beneficial in HE as it allows for reduced intestinal ammonia production and despite limited evidence from randomised controlled trials remain the most widely used therapy for HE.

Non-absorbable disaccharides: It remains unclear how non-absorbable disaccharides exert a beneficial effect. There has been many proposed mechanisms 1) enhanced growth of non-urease producing bacteria,(223) 2) catharsis secondary to bowel acidification reducing ammonia absorption,(224, 225) 3) proliferation of healthy bacteria by providing additional carbohydrate and thus nitrogen (even as ammonia) into protein and/or 4) providing carbon and energy and so spare bacterial ammonia metabolism.(226) More specifically, after consumption lactulose (a sugar) passes through the small bowel completely undigested (unlike glucose, sucrose and lactose, which are easily fermented in the small bowel). Once in the colon, lactulose is fermented by anaerobic bacteria (especially *Bacteroides spp.* (species)) Fermentation of lactulose by colonic bacteria yields important products like the weak acids - Lactic, Acetic & Butyric acid; along with gases like hydrogen (H_2) which modulate intestinal acidity and gas production. This leads to acidification of ammonia into ammonium, which is poorly absorbed. However, physiologically a total daily dose of 10–20g is small compared to 500–1000g faeces/day, such that the impact on acidity/reduced faecal pH on the faecal flora are likely to be limited. This is further suggested by the understanding that Mannitol and Sorbitol both

cause low Ph but neither improve HE.(227) The production of H_2 in the colon may be more important as 1 Litre of H_2 gas is produced from just 7g of lactulose causing flatulence and intestinal hurry, shifting massive amounts of colonic bacteria.(228) However, it may be the provision of energy in preference to ammonia that accounts for the benefit of non-absorbable disaccharides. Furthermore, as lactulose is non-absorbed, it should not have any significant impact on glycaemic control and can be used in diabetic patients.

However, a comprehensive meta-analysis of non-absorbable disaccharides has suggested that previous data from randomised and controlled clinical trials do not support its routine use in clinical practice.(47) There is limited but better evidence from randomised control trials for the treatment of MHE, with lactulose reportedly conferring improved neuropsychometric and quality of life scores,(229) while also preventing recurrence of HE in patients with cirrhosis.(230)The impact of other therapies initiated at the same time often confounds any benefit on HE severity by the established ammonia-lowering effect of non-absorbable disaccharides. Compliance, adverse effects, clinical safely and cost effectiveness are necessary concerns. The limited side effect profile of non-absorbable disaccharides has been at the crux of liberal lactulose prescribing practices. However, it is often overlooked that aggressive use of lactulose causes significant gaseous distension, discomfort and diarrhoea, which may lead to poor compliance. More alarmingly frank dehydration, pre-renal uraemia, hyponatraemia, marked intestinal distension or aspiration of lactulose does occur. Therefore, although non-absorbable disaccharides are relatively cheap, their cost-effectiveness should not be assumed and should be balanced against clinical outcomes that are difficult considering the multi-factorial causes and the differing therapies employed in HE. Therefore we

should be more reserved in our judgment when considering Lactulose as the 'gold standard' for the treatment of HE, justifying placebo-controlled trials of new agents.

***Other purgatives*:** Enemas are beneficial as a means of expelling ammonia producing gut flora by both cleansing and colonic acidification,(231) but are no better than oral purgatives like lactulose. Therefore if bowel motions can be maintained at ≥ 2/day, then enemas may not offer any additional benefit.

Non-absorbable antibiotics

The contribution of intestinal urease-positive bacteria to gut ammonia production is mainly in the colon rather than gastric mucosa (e.g. *Helicobacter pylori*), due to their number and more alkaline colonic pH which favours enhanced ammonia diffusion, such that *Helicobacter pylori* eradication has no therapeutic benefit.(232) Oral, non-absorbable, synthetic anti-bacterial agents such as Neomycin and Rifaximin have been used to inhibit the growth or kill susceptible ammoniagenic bacterial species; showing comparable efficacy to lactulose.(233) Rifaximin is a synthetic antibiotic related to Rifamycin, with wide antibacterial activity against both aerobic and anaerobic gram-negative and gram-positive bacteria. Rifaximin is efficacious, with a superior safety profile and thus preferred to neomycin.(234, 235) However, though used extensively in North America and Europe, rifaximin can only be prescribed on a named patient bases in the UK. Therefore, although not evidence based, non-absorbable antibiotics are reserved for patients who fail to respond to non-absorbable disaccharides.

Modulators of interorgan ammonia metabolism (Figure 1)

The concept of manipulating endogenous biosynthetic pathways to eliminate non–urea waste nitrogen as a substitute for defective urea synthesis is not new.(236) Despite abnormal urea-cycle functioning, decreasing total body nitrogen by promoting the synthesis of non-urea nitrogen-containing metabolites that have high excretion rates appears to be of benefit.

Arginine supplementation: L-arginine is an important dietary substrate for the urea cycle, which allows for ammonia detoxification to urea (via arginase). Unlike the essential BCAA, which can only be derived from ones diet, L-arginine is a semi-essential amino acid, as although metabolically produced, in some disease states may require dietary supplementation. In cases of the childhood urea cycle disorders (e.g. deficiency of carbamyl phosphate synthetase (CPT), ornithine transcarbamylase (OTC), argininosuccinate synthetase (AS) and argininosuccinase (AL)), dietary restriction of L-arginine triggers the rapid development (15-68 hours) of symptomatic hyperammonaemia (e.g. vomiting, lethargy, or irritability).(237) In these disorders there is a significant reduction in urea production, with nitrogen instead accumulating as glutamine (predominately), ammonia and to a limited extent alanine and glutamate. In AS and AL deficiency, provision of additional dietary L-arginine promotes the synthesis of citrulline (1 waste nitrogen atom per molecule) and argininosuccinate (2 waste nitrogen atoms per molecule), allowing for the urinary excretion of nitrogen as urinary waste products; with OTC deficiency, citrulline is preferentially supplemented. The underlying mechanism for this is independent of CPT synthesis, as suggested by orotic aciduria (the direct product of CPT) with arginine deficiency; accumulating substrates driven down alternative

pathways. In acute liver failure (ALF), systemic hypotension and cerebral edema may be associated with increased plasma nitric oxide (NO) levels. L-arginine is the rate-limiting substrate for NO production via nitric oxide synthetase (NOS) but is deficient in ALF due possibly to increased arginase activity in the liver that converts it to urea and ornithine. This may point to a potential benefit for L-arginine supplementation. Yet there have been no good studies evaluating a role for L-arginine supplementation in hepatic encephalopathy, though studies exploring whether correcting L-arginine deficiency alters either portal hypertension or cerebral oedema via either arginase dependent reduction in hyperammonaemia and/or NO/ADMA dependent mechanism(s).

Phenylbutyrate: Phenylbutyrate, which is converted to phenylacetate *in vivo*, is used for the hyperammonaemia associated with urea cycle enzyme deficiencies.(238) Phenylacetate covalently combines with circulating glutamine to form phenylacetylglutamine, which is excreted by the kidneys. In such metabolic disorders characterized by elevated glutamine levels, this excess can be mopped up by phenylacetate, thereby removing glutamine as a substrate for ammoniagenesis. Phenylbutyrate has been trialed in HE associated with liver failure, but with little effect. This probably relates to the need for a high glutamate state, which is absent in liver failure.

Sodium Benzoate: This drug similarly increases the renal excretion of ammonia, but instead excretes it as hippuric acid (hippurate), which contains one waste nitrogen atom per molecule; hippurate being the glycine conjugate of benzoic acid via an amino acid acylation step.(236) Benzoate has been shown to improve the HE that occurs with inborn errors of metabolism.(239) For patients deficient in CPS or

OTC, administration of sodium benzoate stimulates hippurate synthesis and excretion as urinary waste nitrogen. Also, in at least one double-blind randomised control trial was found to be as effective as lactulose in the treatment of acute portosystemic HE.(240)

Combined intravenous sodium phenylbutyrate and benzoate *(Ammonul, Ucyclyd Pharma):* Combined therapy has proved very beneficial for the treatment of urea-cycle disorders. In a recent 25 year open-labeled study, combination therapy lead to a 79% reduction in plasma ammonia and 84% improved survival (dependent on peak ammonia level and age – poor in neonates and 98% in late onset disease).(241) This compares favorably with 16% survival in neonates and 72% with late onset disease in patients without therapy.(242) However, as the N-acyltransferases that conjugate glutamine to phenylacetate and glycine to benzoate are located in the liver and kidney, the severe hapatotoxicity of ALF may eventually lead to response failure, especially with saturation of enzyme capacity (e.g. phenylacetate to PAG).(243, 244)

L-ornithine L-aspartate (LOLA): The concept of delivery of L-ornithine to the muscle is the basis of the agent L-ornithine L-aspartate (LOLA) for ammonia lowering in cirrhosis. LOLA provides L-ornithine and L-aspartate as substrates for glutamate production. There have been a number of clinical studies using of LOLA.(245-247) In a double-blind randomized control study of cirrhotics with mild HE, one week of LOLA reduced ammonia and improved mental function.(245) A cross-over study showed that 20-40g/day of LOLA infusions ameliorated post-prandial increases in ammonia following oral protein loading.(248) However, increased plasma glutamate, unchanged glutamine and increased urea production

with the higher doses(248) conflicts with the muscle ammonia detoxification hypothesis. Notably the 40g dose also induced hyperglycaemia and hyperinsulinaemia.(248) Liver failure models further suggest that LOLA reduces brain oedema of advanced HE.(249) As yet, there are no studies in patients with ALF and its use in ALF is currently not recommended. Critically, there are concerns that the ammonia-lowering effects of LOLA may be only transient, as there are reports of 'rebound hyperammonaemia' and HE recurrence on discontinuing LOLA.(132) This means that though initially ammonia levels fall with infusion of LOLA, soon after its discontinuation it begins to rise back to pre-treatment levels. This could be explained by the significant rise in glutamine levels evoked by the LOLA, eventually becoming a source for ammoniagenesis by the kidney and intestinal (through glutaminase).(133) In addition, aspartate is unlikely to offer an additional beneficial effect as its infusion (in animal models) does not reduce ammonia and thus an unlikely biologically active precursor of glutamate/glutamine.(250)

OTHERS

Acarbose

The hypoglycaemic agent acarbose, which stimulates gut motility, acts through the inhibition of glucose absorption in the gut by promoting intestinal saccharolytic bacterial flora in preference to proteolytic flora, so reducing substrate for ammonia production. In a cross-over randomised trial of cirrhotic patients with low-grade HE and type 2 diabetes mellitus, 8 weeks of acarbose (100mgs TDS) significantly decreased ammonia blood levels, intellectual function, decreased fasting glucose

(33%), decreased postprandial glucose (50%) and significantly lowered glycosylated haemoglobin levels.(251) However, acarbose has not found widespread acceptance for its use in the treatment of HE outside of this select group of patients with co-existent type-2 diabetes mellitus.

Bromocriptine

Bromocriptine, a dopamine agonist, has been used with limited success for disturbances in dopaminergic neurotransmission associated with chronic intractable HE,(252, 253) but such studies failed to show a clear benefit over standard therapy.(254) Furthermore, in cirrhotic patients with ascites it can induce hyponatraemia.(255) However, there is anecdotal evidence to suggest a benefit in a small number of cirrhotic patients with low-grade encephalopathy associated with development of basal ganglia injury and resultant dopamine deficiency.

Correction of precipitating factors (Table 2)

An acute admission with worsening encephalopathy is often precipitated by a number of defined causal factors which can frequently be anticipated and thus pre-emptively corrected, as prompt action may lead to a improvement in HE. Though ACLF may be triggered by such uncommon events as sedatives or tranquilizers, vascular occlusion (hepatic vein or portal vein thrombosis) and hepatocellular carcinoma transformation, it is important to outline the management of the more common precipitants:

- Constipation
- Electrolyte and acid-base imbalance (discussed later)
- Infection (discussed later)
- Gastrointestinal bleeding

- Portosystemic shunts

Constipation: Enemas are beneficial as a means of expelling ammonia producing gut flora either due by cleansing or colonic acidification.(231) However, there is only limited evidence to show a benefit over the use of oral purgatives like lactulose. Therefore if bowel motions can be maintained at ≥ 2/day, enemas are only used as an adjunct to the primarily used non-absorbable disaccharides.

Infections: In addition to that discussed later, bacterial infections predispose to variceal bleeding in cirrhotic patients. A meta-analysis of antibiotic use in variceal bleeding, reported a 30% decrease in rate of infection and 9% improvement in short-term survival.(256, 257)

Gastrointestinal bleeding: Due to the high protein content of blood and thus nitrogenous load, there is increased intestinal ammonia production. This ammoniagenic blood meal and precipitation of HE is potentially related to an absence of the branched-chain amino acid – isoleucine that protects the inhibitory effect of ammonia on the TCA cycle in neuronal cells (see chapter 1.2).

TIPS insertion: The creation of a portosystemic shunt (used to stabilise patients with uncontrolled variceal bleeding or intractable ascites) may induce HE (especially within the first few months). Prophylaxis against encephalopathy with Lactitol (60 g/day) or rifaximin (1200 mg/day) is not proven to be effective during the first month post-TIPS.(258) Therefore careful selection of patients for a TIPS or surgical shunt is necessary.

ACUTE SEVERE HE: INTRACRANIAL HYPERTENSION AND OEDEMA (Table 1)

ALF is characterised by rapid progression of HE to coma stages and is characterised by cerebral oedema and intracranial hypertension, accounting for the 30% mortality rate in ALF.(259) At this point the need to treat the multi-organ effects of ALF, are inseparable from treating its cerebral effects. Early ventilation, intensive care unit admission and judicious use of available therapies have lead to a significant decline in deaths as a result of cerebral oedema. Aiding liver recovery by prompt and specific treatment of the cause of acute liver injury, treating precipitating factors such as dehydration, electrolyte and acid-base imbalance,(140), infection(170) and ameliorating hyperammonaemia remain at the forefront of therapy. The following therapeutic strategies are utilized in the management of severe HE requiring ventilation.

GENERAL

Early airway maintenance is necessary to protect the airway and prevent high carbon dioxide tension and hypoxia which can result in cerebral hyperaemia.(260) Sedation and mechanical ventilation is also essential to safely manage the aggressive HE patients. Once intubated the head should be elevated by 10-20° with minimal intervention and extreme care when moving or turning a patient; to optimise intracranial pressure (ICP) without compromising the cerebral perfusion pressure.(261, 262) Airway protection will also reduce the likelihood of aspiration, pneumonia, defective gas exchange and infection. Sedative requirements (e.g. Fentanyl, Midazolam or Propofol) are low with worsening severity of HE, but are likely to increase with recovery.

Propofol

Propofol is a useful short-acting sedative with a rapid onset of action (within seconds) and very short half-life. In a dose of 6 mg/kg/hr, propofol reduces CBF through decreasing cerebral oxygen consumption and reducing ICP and is of proven benefit in treating refractory seizures. In a study if seven patients with ALF treated with a propofol infusion of 50 µg/kg/min, propofol maintained the ICP within normal limits in six. One patient died from increased ICP and one during OLT.(263) Propofol is also rapidly deactivated via a conjugation reaction both hepatically and extra-hepatically, this non-renal and non-hepatic dependent metabolic clearance makes it a superior sedative agent over benzodiazepines or opioids, as it is less likely to accumulate. It does however have the disadvantage of leading to hypotension.(263)

Circulatory support & fluid management

ALF is a hyperdynamic state with high cardiac output, low mean arterial pressure, and low systemic vascular resistance.(264) Generalized vasodilatation, which produces profound activation of the neurohormonal system, culminates in vasoconstriction of regional vascular beds.(265) Mean arterial pressure should be maintained at a level to keep the cerebral perfusion pressure between 50-65 mmHg.(266) The onset of multiorgan failure often necessitates use of inotropes. Circulatory failure often becomes refractory to inotropes and up to 70% of patients die.(267) A routine short synacthen test on admission to guide the use of steroids is important as adrenal insufficiency is a common complication of ALF.(268)

Renal Support

Renal dysfunction is common (~50-75%) due to either pre-renal, hepatorenal or nephrotoxic (e.g. acetaminophen) causes;(269) usually requires support with

haemofiltration(267) with continuous (compared to intermittent) haemofiltration(270) may avoid rapid water shifts seen with intermittent therapy,(271) to provide greater haemodynamic stability and improved cerebral perfusion pressure.(270, 272) Due to impaired hepatic lactate metabolism, lactate-free dialysates are preferred.(273)

Electrolyte imbalance

Electrolyte imbalance should be corrected aggressively. Hyponatraemia ≤125 mmol/L may precipitate cerebral oedema and is a contraindication for orthotopic liver transplant (OLT).(32, 274) Induced hypernatraemia has been shown to improve ICP and reduce inotropic requirements in traumatic brain injury,(275) and recently was shown to be useful in ALF.(275)

Antibiotic/antimicrobial agent

The incidence of sepsis in ALF is a significant factor in mortality rate and contraindication to transplantation with 75% developing bacterial infection and 30% fungal infections,(276, 277) from the respiratory and urinary tracts, ascites and iatrogenic sources. Administration of broad-spectrum antibiotics/antifungal therapy should be initiated at the first sign of infection, with focused treatment once the organism is identified. Despite the absence of randomised control trials of prophylactic antimicrobials in ALF, the use of systemic antibiotics in ALF is widespread.(25, 28)

Glycaemic control

Both hyper and hypoglycaemia need rapid correction as they may worsen brain oedema. The role of tight glycaemic control in ALF has not been ascertained but

must be instituted with caution because of the tendency for development of hypoglycaemia.

SPECIFIC

Mannitol

Mannitol (an osmotic diuretic) increases brain capillary osmolality, drawing water from the brain tissue into the capillaries and has been shown to significantly reduce the extent of cerebral oedema and improve survival.(278, 279) Bolus doses of 20% mannitol at 1g/kg are preferred. Plasma osmolality should be kept <320 Osm/l. If patient is oligouric, mannitol may accumulate and can only be used with concomitant haemofiltration.

Dexamethasone

In ALF, reducing inflammation (whether systemic or local) by utilizing the anti-inflammatory effects of steroids may improve cerebral haemodynamics and prevent/treat intracranial hypertension.(28, 29, 280) However, trials using dexamethasone in advanced ALF have shown little effect on the frequency of cerebral oedema or survival.(278)

Mild hypothermia

Induced hypothermia may act on multiple pathogenic mechanisms involved in development of HE. For many years induced hypothermia has been used to limit the development of intracranial hypertension associated with head injury. In animal models of ALF the use of hypothermia has been demonstrated to significantly reduce brain water, duration of encephalopathy and improve outcome compared to

euthermic animals.(183, 281, 282) Using cooling blankets to induce moderate hypothermia (target core temp. 32–33°C) can lead to a reduction in ICP; even in patients unresponsive to mannitol and/or ultrafiltration and acts as a bridge to OLT.(146, 156) Hypothermia also significantly improves cardiovascular haemodynamics manifested by increased mean arterial pressure and systemic vascular resistance with reduced noradrenaline requirements.(156)The mechanisms underlying the protective effect of mild hypothermia may relate to a reduction in arterial ammonia and also brain ammonia extraction and flux.(116, 146) As yet, there is no data from randomized control trials on the use of hypothermia in ALF, but its use is widespread in patients with uncontrolled intracranial hypertension.

Thiopental sodium

By inducing cerebral vasoconstriction through inhibition of NOS, Intermittent bolus injections (1.5-3.5 mg/kg) or continuous infusion of thiopental, reduce elevations of ICP.(283) However, its use is limited to intractable increases in ICP unresponsive to other therapies, because of profound negative effects on systemic haemodynamics like reduced mean arterial pressure and thereby cerebral perfusion pressure.

Indomethacin

Non-steroidal anti-inflammatory (NSAIDS) may modulate brain function(284) via effects on multiple inflammatory pathways (e.g. antipyretic), as well as possible direct effects on cognitive function (via modulation of the glutamate-nitric oxide-cyclic GMP pathway),(285) and via effects on prostanoids regulation of cerebral vascular tone and cerebral blood flow (CBF). Indomethacin, a nonselective cyclooxygenase (COX) inhibitor,(286) when given at a dose of 0.5mg/kg in selected patients can reduce ICP and cerebral oedema independent of a change in cerebral

blood flow.(287) However, its use is limited by nephrotoxicity, platelet dysfunction and risk of gastrointestinal bleeding. Poor brain penetration of NSAIDs at therapeutic levels requires high doses which increases the risk of toxicity.(285, 288)

Anti epileptic drugs (AEDs)

In a randomised study of 42 ALF patients with grade 3-4 HE, subclinical seizures were less frequent with the use of the anti-epileptic drug Phenytoin, which acts upon the Na/K ATPase, and also limit development of ICP and cerebral oedema ($p<0.03$);(289) though this is contended in a recent study from India.(290) However, caution should be taken as Phenytoin is hepatically cleared and with a failing liver its clearance may become saturated, shifting the elimination from first- to zero-order kinetics and predisposing to intoxication.(291) In this situation even extracorporeal elimination may prove difficult due to the high level of protein binding of phenytoin. Also the associated hypoalbuminaemic state with liver failure, even if the total phenytoin concentration is only moderately elevated, its free fraction may be very high;(291) again potentially causing severe neurologic signs of phenytoin intoxication (e.g. sedation, cerebellar ataxia, ophthalmoparesis, and paradoxical seizures).

N-acetylcysteine (NAC)

In a case of acetaminophen overdose, NAC must be continued irrespective of the time between the overdose and presentation and acetaminophen level as it can prevent progression to fulminant hepatic failure and reduces mortality (58% vs. 38%) especially in those who progress to grade III–IV HE (51 vs. 75%).(292) There is less convincing evidence for NAC in non-acetaminophen overdose (20% vs. 48%).(293, 294) In non-acetaminophen ALF, NAC may improve survival by its

effects on cardiac output, oxygen extraction and consumption, and due to its anti-oxidant effects that ameliorate the significant oxidative stresses that occurs with liver failure.

Flumazenil

In a large placebo controlled trial focusing on intensive care patients with advanced HE (grade III-IV), the short-acting benzodiazepine-receptor antagonist flumazenil was shown to rapidly improve the neurological score in 15% and electroencephalogram (EEG) findings in 30% of patients within minutes of its administration.(295) However, flumazenil does not lead to any lasting effect or correct HE, unless co-administered with a long-acting therapy,(296) and as such is not recommended.

Liver support and transplantation

Orthotopic liver transplantation: Although OLT offers definitive therapy for liver failure by dramatically improving the clinical status with a return to a normal mental state, there is evidence to suggest that minimal HE may persist in certain patients due to some as yet unknown irreversible changes in the brain.(173) The incongruity that exists between donor organs and recipients has lead to a plethora of extracorporeal liver assist devices (ELADs)(297, 298) and even partial hepatectomy(29, 196) to aid or supplant the failing liver. ELAD's are either:

- *Biological (including hybrid & combination) devices:* which use either immortalised hepatocytes cultured in bioreactors or whole animal livers to mimic endogenous excretory and synthetic liver function.

- *Non-biological devices:* which use extracorporeal blood purification to dialyse albumin-bound hydrophobic substances (e.g. ammonia, bilirubin, bile acids, aromatic amino acid metabolites and medium-chain fatty acids).

The extracorporeal devices under clinical evaluation include the following:

***Molecular Adsorbent Recirculating System (MARS)*:** provides counter-current haemodialysis against albumin and bicarbonate circuits.(297)

***Single-Pass Albumin Dialysis (SPAD)*:** provides counter-current albumin dialysis against high flow blood in a fibre haemodiafilter, which unlike MARS is discarded after passing the filter. As it uses a standard renal dialysis device continuous veno-venous haemodiafiltration is possible.(299)

Prometheus system: provides direct albumin adsorption with high-flux haemodialysis after selective filtration of the albumin fraction through a specific polysulfon filter.(300)

All these devices successfully remove protein-bound toxins, but may have more variable effects on systemic (versus portal) haemodynamics; and worsen coagulopathy. Currently the clinical benefit of such devices is unclear, although they may offer a bridge to transplantation or liver recovery.

In conclusion, for HE, ammonia-lowering therapies remain the cornerstone of standard medical care for this debilitating condition, along with wider measures to treat precipitating factors and the specific interventions for the cerebral sequelae of

advanced disease. As highlighted in chapter 1.3, knowledge garnered from a greater understanding of interorgan ammonia and amino acid metabolism and the pathophysiological basis of HE are most likely to lead to the development of new therapeutic approaches.(133) However, even current best treatment(s) lack conclusive evidence from clinical studies to-date,(47, 48) such that with the advent of new (and existing drugs) there is a requirement for robust randomized controlled trials to drive an evidence based approach to the treatment of HE.

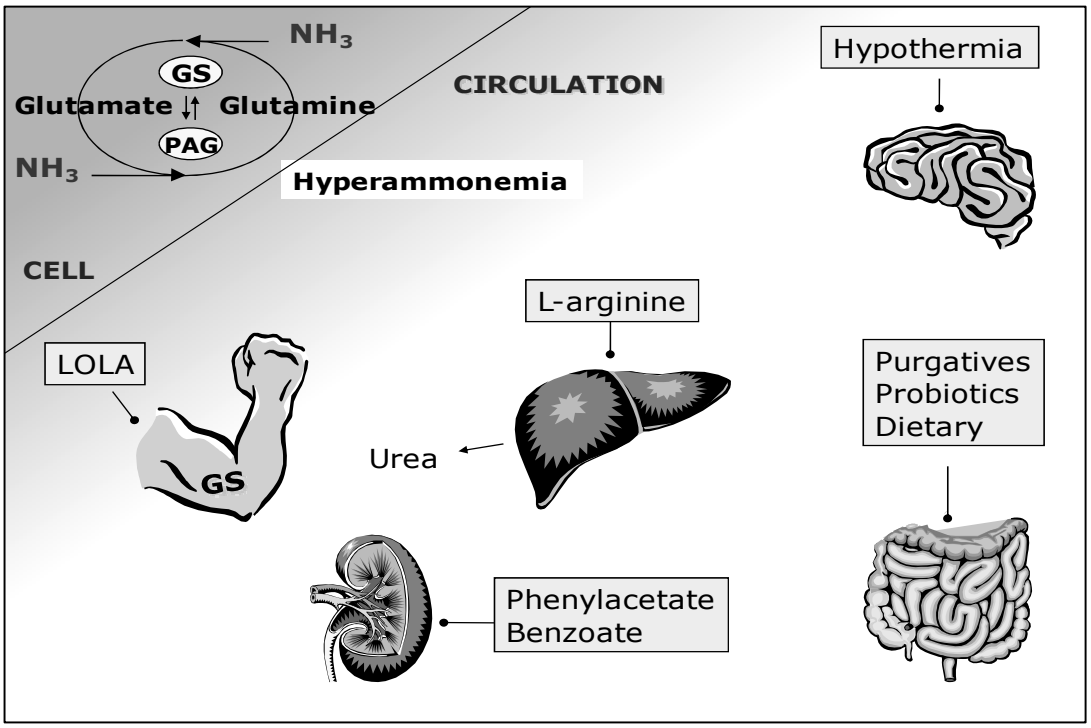
Table 1: Treatment stratagems used in HE

HE grade: I-II	HE grade: III-IV
General management	**Cerebral oedema & elevated ICP**
HYPERAMMONEMIA **Dietary protein supplementation** **Purgatives** • Non-absorbable disaccharides • Enemas **Non-absorbable antibiotics** **Modulation of interorgan ammonia** • L-ornithine, L-aspartate (LOLA) • Sodium benzoate • Phenylacetate **Others** • Flumazenil, Bromocriptine, Acarbose **EMERGING THERAPIES** • Probiotics	**General** • Ventilate • Sedate (e.g. Propofol) **Specific** • Antimicrobials • Hypertonic saline • Mannitol • Dexmethasone • Induced hypothermia • Thiopentone • Indomethacin • Anti-epileptic drugs • N-acetylcysteine (NAC)
	Transplantation
	Orthotopic liver transplant (OLT) **Partial hepatectomy** **Liver assist devices**

Table 2: Precipitating Factors In Hepatic Encephalopathy

Precipitating Factors In HE
Constipation
Dehydration
Gastrointestinal bleeding
Infection
Excessive dietary protein
Hypokalaemia
Hypoglycaemia
Hypothyroidism
Hypoxia
Metabolic alkalosis
Anaemia
Azotaemia/uraemia
Medications (narcotics, sedatives, etc.)
Hepatoma
TIPS, surgical shunt
Vascular occlusion

Figure 1: Therapies - interorgan ammonia and amino acid metabolism



In liver failure, the relative activities of cellular glutamine synthetase (GS) and phosphate-activated glutaminase (PAG) in different organs influence interorgan ammonia and amino acid metabolism. With a loss of hepatic urea cycle capacity, hyperammonaemia is predominately due to worsening intestinal and renal ammonia efflux, with skeletal muscle having the potential to increase its ability to detoxify ammonia. Though the brain also detoxifies ammonia, this is counterproductive as resultant astrocyte glutamine accumulation induces brain swelling. This schematic highlights not only current standard therapies for hyperammonaemia, which principally act on individual organs (e.g. purgatives targeting intestinal ammonia production), but also newer interventions targeting multiple organs (e.g. LOLA).

Chapter 2

Hepatic Encephalopathy:

Mechanistic study -

Role of ammonia & inflammation

Published in '**Hepatology**' (2007)

Title:

"Endotoxemia produces coma and brain swelling in bile duct ligated rats"

Authors:

Wright GA, Davies NA. Shawcross, DL. Hodges, SJ. Zwingmann, C. Brooks, HF. Mani, AF. Moore, KP. Harry, D. Stadlbauer, V. Zou, Z. Williams, R. Davies, DC and Jalan, R.

Abstract

This study explores the hypothesis that the inflammatory response induced by administration of lipopolysaccharide (LPS) exacerbates brain oedema in cirrhotic rats with cirrhosis; and if so whether this is associated with altered brain metabolism of ammonia or anatomical disturbance of the blood-brain barrier. Adult Sprague-Dawley rats 4-weeks after bile-duct ligation (BDL)/sham-operation, or naïve rats fed a hyperammonemic diet (HD) were injected with LPS (0.5 mg/kg, I.P) or saline and sacrificed 3-hours later. LPS administration increased brain water in HD, BDL and sham-operated groups significantly ($p<0.05$), but this was associated with progression to pre-coma stages only in BDL rats. LPS induced cytotoxic brain swelling with maintained anatomical integrity of the blood-barrier. Plasma/brain ammonia levels were higher in HD and BDL rats than sham-operated controls, and did not change with LPS administration. Brain glutamine/myo-inositol ratio was increased in the HD group but reduced in the BDL animals. There was a background proinflammatory cytokine response in the brains of cirrhotic rats, and plasma/brain TNF-α & IL-6 significantly increased in LPS-treated animals. Plasma nitrite/nitrate levels increased significantly in LPS groups compared with non-LPS controls but frontal cortex nitrotyrosine levels only increased in the BDL + LPS rats ($p<0.05$: versus BDL controls). In conclusion, injection of LPS into cirrhotic rats induces pre-coma and exacerbates cytotoxic oedema due to the synergistic effect of hyperammonaemia and the induced inflammatory response. Although the exact mechanism of how hyperammonaemia and LPS facilitate cytotoxic oedema and pre-coma in cirrhosis is not clear, this data supports an important role for the nitrosation of brain proteins.

Background

Hepatic encephalopathy (HE) is an important complication of cirrhosis, the severity of which can vary widely from mild sleep disturbance through neuropsychological impairment to coma, brain herniation and death. Though a common, and defining characteristic of acute liver failure (ALF),(301) the development of advanced cerebral oedema in cirrhosis is thought to be rare. However, severe brain swelling and intracranial hypertension has been reported in patients with cirrhosis who have severe precipitating illness, such as uncontrolled gastrointestinal bleeding, insertion of a transjugular intrahepatic shunt and/or severe sepsis.(32, 302, 303)

Although the exact pathogenesis of HE is not clear, ammonia is thought to play a central role and its levels have been shown to correlate with the severity of HE in cirrhotic patients.(23) Furthermore, arterial ammonia levels predict brain herniation in patients with ALF.(22) Ammonia is thought to produce astrocytic brain oedema through the accumulation of glutamine which is the detoxification product of ammonia metabolism resulting in increased osmotic stress.(150, 304) Indeed, extracellular glutamine levels were shown to correlate with the severity of intracranial hypertension.(147) Yet comparatively, in cirrhosis *Myo*-inositol (a sugar involved in phosphoinositide synthesis) is reduced with increasing concentrations of glutamine; some investigators suggesting that *myo*-inositol and other weaker intracellular osmolytes are expelled from the cell to compensate for the entry of glutamine.(305)

In patients with ALF, the severity of inflammation (often secondary to infection) correlates with the progression to advanced stages of encephalopathy and intracranial hypertension.(26, 28) Recently, our group reported a correlation between the severity of increased intracranial pressure and the circulating levels of proinflammatory cytokines in ALF patients.(169) Brain cytokine production has also

been observed in a group of ALF patients with uncontrolled intracranial hypertension.(10, 169) In patients with cirrhosis, it has been shown that induced hyperammonaemia results in significant worsening of neuropsychological function in those patients with evidence of an inflammatory response due to bacterial infection. This effect was not found after resolution of the infection following antibiotic therapy.(170)

These data strongly indicate a possible synergy between hyperammonaemia and inflammation in the development of HE, but it is not clear whether it is the background cirrhotic state or the associated hyperammonaemia that predisposes to the effects of the superimposed inflammation. This raises a number of lines of investigation:

Research Hypothesis

On a background of chronic liver failure, systemic inflammation and hyperammonaemia act synergistically to sensitise the brain to their effects and allow for advancing encephalopathy.

Research questions

5. Do systemic changes in ammonia and inflammation act synergistically to effect the progression and complications associated with HE as indicated by the depth of coma or cerebral oedema?
6. With progression of HE, is there an anatomical or functional alteration of the blood brain barrier?
7. Does the LPS challenged BDL rat model represent clinical ACLF?

8. On a background of hyperammonaemia and systemic inflammation, are the cerebral effects associated with HE due to alterations in brain ammonia and amino acid metabolism, and/or cerebral inflammatory responses?

Aim of study

Based on the above questions, it was necessary to undertake the following study to explore the hypothesis that the inflammatory response induced by administration of bacterial cell wall LPS, exacerbates brain oedema in cirrhotic rats; and if so whether the oedema is associated with altered brain metabolism of ammonia or anatomical disturbance of the blood-brain barrier. I chose to perform this study using our well-characterised BDL model of cirrhosis and diet induced hyperammonemic rats.

Methods

All animal experiments were conducted according to Home Office guidelines under the UK Animals in Scientific Procedures Act 1986. Male Sprague-Dawley rats (body weight 230–280g) were obtained from the comparative biological unit at the Royal Free and University College Medical School, University College London. All rats were housed in the unit and given free access to standard rodent chow and water, with a light/dark cycle of 12 hours, at a temperature of 19–23°C and humidity of approximately 50%.

Animal models

Bile-duct Ligation: Under anaesthesia (diazepam 1mg/kg intravenous (I.V), followed by Hypnorm® 150µl/kg intramuscular (I.M), Janssen Pharmaceutica, Belgium) all rats underwent bile duct ligation to induce biliary cirrhosis, or a sham operation as described previously.(306) One BDL and 1 sham-operated rat died within 36 hours of the operation due to anaesthetic complications.

Non-cirrhotic hyperammonemic animals: Naïve rats (n=14) were administered a high protein/ammoniagenic diet (HD) for 7 days prior to administration of LPS/Saline 3 hours before termination (as outlined above). The diet consisted of a liquid rodent feed (Bioserve, Frenchtown, NJ 08825, USA) and a tailor-made mixture mimicking the amino-acid composition of haemoglobin molecule (4g/Kg/day Nutricia, Cuijk, The Netherlands) as described previously(115, 307) mixed with commercially available gelatin to prevent sedimentation. This regimen produces chronic hyperammonaemia to levels that are similar to that observed in BDL animals.

Study Design

Twenty-eight days after surgery, the operated rats (BDL and sham) were randomised into four groups, with BDL or sham-operated rats injected with LPS

(*Salmonella typhimurium spp*., 0.5 mg/kg I.P made up to 0.5ml in saline or saline alone: 1) Sham-operated + saline (n=7), 2) Sham-operated + LPS (n=7), 3) BDL + saline (n=6) and 4) BDL + LPS (n=6). The HD rats were also randomised to receive LPS (n=7) or saline (n=7).

As per protocol, the rats were allowed free access to food and water (*ad lib*) for a period of 3 hours post-intervention in a temperature controlled environment and were then sacrificed by exsanguination under anaesthesia (Hypnorm™ 200µL/kg I.M), 20 minutes after diazepam (1mg/kg I.P). In all study groups over this 3 hour post-intervention period, food intake was minimal, likely reflective of the effect of anaesthesia. Blood was withdrawn from the descending aorta and immediately put into ice cold heparin/EDTA containing tubes (until full exsanguination), centrifuged at 4°C, and the plasma collected and stored at -80°C until assayed.

Assessment of level of consciousness

The conscious levels of rats used in this study were rated using an established neurological scale.(308) It was rated as either normal, with loss of the scatter reflex (c.f. Grade 1 encephalopathy) and ataxia (c.f. Grade 2 encephalopathy), together representing pre-coma stages, or loss of the righting reflex (c.f. Grade 3-4 encephalopathy) representing the coma stage. In a previous study(306) it was established that 3 hours after administration of LPS, BDL rats all reached pre-coma or early coma stages. Therefore, in the current experiments all rats were sacrificed 3 h after injection of LPS or saline.

Brain water measurement

Though many distinct areas of the brain demonstrate perivascular oedema, with certain areas now considered to have a more direct associated with neurobehavioural change at non-overt HE (e.g. anterior cingulate cortex(309)) the 'frontal' cortex was chosen as the primary area of interest based on our own

experience and prior validated studies(37, 310, 311) as it displays consistent oedema and correlative coma and alterations in amino acid concentrations with injury.

At the time of death, the whole brain was rapidly removed and 2mm^2 samples were dissected from the frontal cortex (grey matter) and the cerebellar cortex. Brain tissue water content (as a measure of cerebral oedema) was determined using a previously described gravimetric technique.(312) Tissue samples were coded with separate investigators (Dr Gavin Wright, Dr Nathan Davies, Dr Stephen Hodges, Dr Vanessa Stadlbauer) collecting tissue samples or, performing gravimetry (in adjoining rooms).

The gravimetric technique, illustrated in Figure 3, allows the percent gram water per gram tissue of samples less than 2 mm^3 to be determined within minutes to an accuracy of greater than 1%. A modified technique was used; - two mixtures of kerosene and bromobenzene were used in the preparation of the gradient. One litre of mixture A was prepared from 278 ml of kerosene and 100 ml bromobenzene to give a specific gravity of 0.9750. One litre of mixture B was prepared from 279 ml kerosene and 180 ml of bromobenzene to give a specific gravity of 1.0650. A flask containing 100ml of mixture B was placed 40 cm above an empty 100ml graduated cylinder. 100ml of mixture A was then placed 43cm above mixture B.

The fluid kinetics of this system is such that a linear gradient column is produced if the outflow from the constantly mixed 'Mixture B' to the graduated cylinder is exactly twice the outflow from A to B. This was accomplished by using two equal lengths of polyethylene outflow tubing of length 80cm and internal diameter 0.76mm were used and bound together (with tape 3cm from the end) extending from flask B to the graduate and a further 80 cm single length of tubing from Flask A to Flask B. By this technique, the specific gravity at the very bottom of the

graduated cylinder is equal to that of Mixture B (1.065), while the specific gravity at the top is equal to the arithmetic mean of the two solutions (1.020). A steady flow to the surface of the graduated cylinder was maintained by gradually lowering the cylinder in 2 mm increments as the fluid level increased. When a cylinder volume of 100ml was reached the tubes were clamped. The gradient was then permitted to stabilise for 15 minutes and calibrated with standards made up of potassium sulphate (K_2S0_4) of known specific gravity. The concentrations of the K_2S0_4 solutions were 2.99g, 2.67g, 2.35g and 1.7g/50ml (50g of $_dH_20$), corresponding to specific gravities of 1.045, 1.040, 1.035 and 1.025. One drop of each standard was gently placed in the column by means of a small syringe. The depth of equilibration was recorded at the end of 2 minutes. The forebrain and cerebellum was cut into 2 mm3 pieces and placed gently in the column by needle tip. The depth of equilibration was recorded at the end of 2 minutes. 6 pieces for each brain area were recorded. The specific gravity of the samples was calculated using the gradient of the line (y= mx + c) generated from a graph of the specific gravity of the K_2S0_4 standards (x axis) and the column position (y axis). Using a constant (c) to represent the specific gravity of the solid component (1.298 in rat frontal cortex and 1.269 in rat cerebellum) the percentage brain water can be calculated using the following formula: [c/(c-1)] / SpG] – [1/(c-1)] x 100%

Measurement of plasma biochemistry

Plasma samples (200µl) were analysed for: ALT, albumin, total protein, ammonia, bilirubin, urea and electrolytes using a Cobas integra 400 multianalyser with the appropriate kits (Roche-diagnostics, Burgess Hill, West Sussex UK).

Measurement of ammonia

Plasma: Ammonia was measured in the deproteinised plasma using the well validated indophenol detection method as described previously.(313) In brief,

plasma samples were deproteinised by the addition of trichloroacetic acid (15% final) and centrifuged at 10,000 rpm for 10 minutes at 4oC. The supernatant (50µl) was incubated in a 96 well plate at 30^{o}C for 3 hours in 200µl of a solution containing phenol (10mM), nitroprusside (10mM), sodium hypochlorite (10mM) and sodium hydroxide (0.5mM). The concentration was determined against a standard curve measured at 630nm in a 96 well plate reader as above.

Brain: The cerebral cortex (100µg) was homogenised and deproteinised (using a glass tube Teflon pestle homogeniser) in 300µl of ice-cold cell lysis buffer solution. After centrifugation at 12,000g for 10 minutes at 4°C, the supernatants were collected, and ammonia measured using absorbance photometry (Cobas integra 400 multianalyser; Roche diagnostics, UK).

Brain Proton magnetic resonance spectroscopy (^{1}H-MRS)

Snap frozen cortical brain samples were processed and analysed by ^{1}H-NMR using a previously described technique (by Dr Claudia Zwingmann, Germany).(38) ^{1}H-NMR spectra were recorded on a Bruker WB 360 spectrometer using a 5-mm QNP probe, 100-200 accumulations, repetition time 16 s, spectral width 3623 Hz, data size 16 K, zero filling to 32 K. Chemical shifts were referenced to lactate at 1.33 ppm. Total metabolite concentrations (µmol/g ww) were analysed from ^{1}H-NMR spectra of PCA extracts using (trimethylsilyl)propionic-2,2,3,3d$_4$-acid as external standard.(314)

Measurement of Nitrite/nitrate

Combined NO_2/NO_3 levels were determined from the heparinised plasma samples by a modified Greiss test, which involves colorimetric detection with Griess reagents, using methods described previously (by Dr Nathan Davies at the Institute of Hepatology, UCL).(175, 315, 316) The modified assay used combined reduction of nitrate and measurement of nitrite in a single step. Reduction was achieved with vanadium(III), which has a lower toxicity than previously used cadmium and did

not require removal prior to nitrite measurement. Vanadium(III) can be used to reduce nitrate to NO at >80°C for chemiluminescent detection, but at lower temperatures, nitrate reduction by vanadium(III) is halted following nitrite formation. Trapping with Griess reagents eliminates the need for chemiluminescent detectors, giving simultaneous detection of nitrate and nitrite in microtitre plate format. In this study plasma samples were diluted and centrifugally separated to remove proteins though a 12-kd cut-off filter (Vectaspin, Whatman, Maidstone, UK). The nitrite and nitrate in the filtrate were determined against a standard curve measured at 550 nm as above, utilizing methods described by Miranda et al.(316) Nitrite concentrations were measured in the filtrate by chemiluminescence as described previously.(306) In brief, sample aliquots were injected into an anaerobic (Argon-purged) reaction vessel containing either Sodium iodine (NaI) in glacial acetic acid (nitrite reduction) or hot vanadium (III) chloride (VCl_3) in Hydrogen chloride (95°C; nitrite and nitrate reduction). The resulting NO was drawn by vacuum into the detector, where it reacted with O_3. This chemiluminescent reaction was quantified and integrated with a photomultiplier tube/computer system.

Measurement of brain tissue nitrotyrosine levels

Brain tissue was homogenised in buffer and proteins precipitated by mixing with methanol/chloroform (1:2). Brain tissue homogenates were analysed for nitrotyrosine (NT) and tyrosine levels which were quantitated using a stable isotope-dilution gas chromatography/negative ion chemical ionisation mass spectrometry method (by Dr A Mani and Dr Kevin Moore at the Royal Free Hospital Medical School, UK).(317-319) This method prevents the artifactual nitration of tyrosine that occurs during acidic hydrolysis conditions. This method has an intra-assay and inter-assay variation of less than 5%. All results are expressed in relation to dry weight of protein as measured by direct weighing prior to hydrolysis.

Measurement of plasma & brain cytokine levels

Snap frozen and stored (-80°C fridge) 100µg cortical brain samples were homogenised cell lysis buffer solution. Following protein concentration quantification of brain homogenates, equilibrated brain protein samples (50µl) were loaded onto TNF-α (Bender MedSystems; Vienna Austria), IL-1β (Biosource International Inc., Nivelles, Belgium) and IL-6 (Biosource International Inc., Nivelles, Belgium) ELISA 96 well plate kits and read using a 96 well plate reader at 450nm (Sunrise, Tecan, Salzburg, Austria) according to manufacturer's instructions.

Histopathological assessment

A further 24 rats (n=4) BDL + LPS, (n=4) BDL + saline, (n=4) sham-operated + LPS, (n=4) sham-operated + saline, (n=4) HD and (n=4) HD + LPS) were anaesthetised for brain tissue collection (Diazepam IP 1mg/kg: Hypnorm, IM. 200µL/kg). Following an extended thoracotomy the rats were perfused with a heparinised saline rinse before administering fixative through the left ventricle, preceding superior vena cava puncture. A perfusion system was used which incorporated a manually controlled hand-held sphygmomanometer to maintain a consistent perfusion pressure (between 100-110mmHg) and thus tissue perfusion to limit vessel collapse, which may occur with venosection. The fixative (a 2.5% gluteraldehyde and 2% paraformaldehyde in 0.1M coccodylate buffer Ph 7.2) contained 2% Lanthanum nitrate (Agar Scientific Ltd, U.K), a low molecular weight (MW 139.8) ionic tracer used to evaluate the integrity of the blood brain barrier. Following removal, the brain was stored in fixative and refrigerated at 4ºC until processing for microscopy (performed primarily by Dr G Wright, assisted by Professor DC Davies, Dr Raymond Moss and Dr HF Brooks at The Department of Anatomy, St Georges University Medical School). 2 mm^3 sections of cerebellar and frontal were processed into Spur's resin (Agar Scientific Ltd, U.K). Briefly, selected

pieces of tissue were rinsed in coccodylate buffer and post fixed in 1% osmium tetroxide in coccodylate buffer (0.2 M, pH7.2). After re-rinsing in buffer, the tissue was dehydrated in ascending alcohols (50%, 70%, 90%, 95% and 100%), before being immersed in propylene oxide and finally embedded in Spur's resin. Semi-thin (1-2μm) sections were cut, stained with 1% toludine blue and viewed in a light microscope to determine the orientation of the tissue blocks. Selected sections of the cortex were picked up and placed on copper grids, stained with aqueous uranyl acetate(18) and washed with distilled water followed by Sato's lead citrate(19) for 20 min each. The sections viewed were then viewed in a Hitachi 7100 transmission electron microscope at 75kV. Digital images were captured with a Gatan column mounted CCD camera at a resolution of 1024 x 1024 pixels and archived on a personal computer. All materials and reagents were obtained from the Sigma chemical company (Poole, UK) unless otherwise stated. All materials and reagents were of a suitable laboratory grade and obtained from the Sigma chemical company (Poole, UK) unless otherwise stated.

Statistics

Data are expressed as mean (±SEM). Significance of difference was tested with ANOVA, the unpaired t-test and Mann Whitney test, indicated in figure legends; $p<0.05$ was taken to be statistically significant. Software used included Microsoft Excel 2003 (Microsoft Corp., Redmond, WA) and GraphPad Prism 4.0 (GraphPad software, Inc., San Diego, CA).

Results

All rats continued to gain weight following surgery. From the final weight taken immediately prior to termination, BDL rats (mean of 320g; n=12) were found to be marginally heavier compared to sham-operated controls (mean of 296g; n=14); p=0.01. This difference was attributed to the presence of ascites. At 28 days, all BDL rats were deeply jaundiced. Although there is a reported anorexic effect with BDL, this was not evident in this study with daily intake of feed no different between groups.

Any potential shock was reduced as the study rats were used to handling and because of the anaesthetic protocol used. This was indicated by the maintenance of systemic hemodynamics seen within our study groups (MAP, Mean ± SEM: sham-operated, 118 ± 3.83; BDL 104 ± 6; HD 132 ± 7; p >0.5), with the effect of LPS on hemodynamics (over 3 hours) already well characterised by our group.(320)

Conscious level and Brain water

All rats were still alive 3 hours after injection of LPS or saline. Following injection of LPS, the BDL rats had a progressive, and marked deterioration in their conscious level starting within 30-60 minutes, reaching pre-coma stages at the 3-hour sacrifice time point. The BDL, sham-operated and HD rats administered saline and the sham-operated and HD rats administered LPS remained fully alert. There was a significant increase in the water content in the frontal cortex of rats administered LPS compared with the corresponding saline-administered controls (sham-operated, p=0.01; BDL, p=0.01; and HD, P=0.05: Table 1, Figure 4). There was no significant difference in the brain water content between saline-treated sham-operated and BDL groups (p=0.237). Compared to sham-operated saline-administered controls, frontal brain water was significantly increased in BDL + LPS (p=0.01), HD (p=0.001) and HD + LPS (p=0.001) groups. There was no significant difference in

the brain water content between the LPS-administered groups. There was no effect of LPS on cerebellar water content in any of the groups studied (data not shown).

Biochemistry

In BDL rats, ALT, bilirubin and albumin levels were consistent with cirrhosis (Table 2) and the bilirubin was significantly elevated compared to sham-operation and HD groups ($p<0.001$), respectively. There was no significant impact following LPS administration in BDL, sham-operated or HD rats except for an expected, and previously well described, rise in ALT and urea seen in the BDL rats. Plasma glucose and albumin were lower in the BDL animals, but the renal function, sodium, potassium and plasma osmolarity were not significantly different between groups (Table 2).

Ammonia

Compared to sham-operated saline control rats, there was a significantly elevated arterial ammonia level in BDL ($p=0.019$), HD ($p=0.049$) and HD + LPS challenged ($p=0.042$) rats (Figure 5, Table 1); significance between two groups established using T-test analysis. There was no significant difference found between BDL and HD saline-treated groups ($p=0.237$). The brain ammonia concentration was found to be elevated in BDL and HD rats compared with sham-operated rats. LPS administration did not alter brain ammonia concentrations significantly from the respective saline-treated animals (Table 3).

Proton NMR spectroscopy

There was no statistical difference in brain tissue osmolytes following administration of LPS to any of the test groups compared to the saline control (Table 3, Figure 6). There was a significant decrease in brain glutamine levels in BDL compared to sham ($p<0.03$), and significant increase compared to HD ($p=0.01$) saline-treated rats. A significant decrease in brain *Myo*-inositol levels was found in BDL + saline and HD

(±LPS/saline) rats compared to sham-operated rats (BDL, p=0.01; HD + LPS, p=0.005; and HD, p=0.014, respectively). Also, the brain glutamine/*myo*-inositol ratio was significantly higher in our HD (±LPS/saline) rats compared to sham/BDL ±LPS/saline rats (p<0.001 for each case).

Plasma and Brain Cytokines

Following administration of LPS in sham-operated and BDL rats there was a significant rise in the plasma levels of the proinflammatory cytokines TNF-α and IL-6 (p<0.001: compared to each corresponding saline control group), which was also observed in HD rats (p<0.05, Table 4). In saline-treated BDL rats, the levels of brain IL-6 and TNF-α were increased 1.6- and 1.5-fold respectively compared to the sham-operated group; though this only reached significance for measured IL-6 concentrations, most likely due to intra-group variation (p=0.05, unpaired t-test). This background inflammatory response, in which brain TNF-α was augmented by 5-fold following administration of LPS in BDL rats, was found to be not statistically significant.

Nitrite/Nitrate (NOx)

The plasma concentration of NOx (Figure 7A, Table 1) was significantly higher in rats treated with LPS compared to the non-LPS-administered groups (sham-operated, p=0.017; BDL, p=0.008 and HD, p=0.05, respectively). In saline-treated controls, BDL rats also had higher NOx levels compared to sham-operated controls (p=0.05). Similarly, the BDL, sham-operated and HD rats treated with LPS also had higher plasma nitrite values compared to their respective saline-treated control rats (sham-operated, p=0.045, BDL, p=0.038 and HD, p=0.05: Figure 7B: Table 1).

Brain Nitrotyrosine/Tyrosine ratio

The only significantly increased NT/T ratio was observed in the LPS-administered BDL rats compared to saline-treated BDL controls (p<0.05: Figure 7C, Table 1);

calculated using the Newman-Keuls Multiple Comparison Test (to determine differences between all groups). The administration of LPS had no significant effect on sham-operated or HD rats.

Electron Microscopy

Figures 8A-F: are transmission electron micrographs (Scale bar, 2 μm) showing representative cerebral microvessels and the perivascular astrocytes for each treatment group.

Sham-operated rats: Figure 8a, shows a fully intact and well-perfused microvessel with no surrounding astrocytic, perivascular changes. Lanthanum nitrate particles line the microvessel luminal wall, suggesting an intact blood brain barrier to this tracer.

BDL rats: Figure 8b, shows a partially collapsed microvessel, although the endothelium barrier appears intact due to the fact that the lanthanum particles are clearly retained in the microvessel lumen. There is only minimal brain water accumulation observed in the astrocytic, perivascular tissue.

Sham-operated + LPS rats: Figure 8c, shows mild astrocytic, perivascular oedema despite an otherwise intact blood brain barrier as suggested by the retained lanthanum.

BDL + LPS rats: Figure 8d, shows greater disruption of structure than found in the other *sham* and BDL experimental groups, evident by the massive astrocytic, perivascular oedema and collapsed microvessel. However, the blood brain barrier still appeared intact with no leakage of the lanthanum nitrate into either the endothelial cell cytoplasm or perivascular space.

HD rats: Figure 8e, shows a partially collapsed microvessel, although the endothelium barrier appears intact due to the retention of the lanthanum particles

in the microvessel lumen. There is massive brain water accumulation in the astrocytic, perivascular tissue.

HD + LPS rats: Figure 8f, shows similar disruption of structure to that found in BDL + LPS and HD rats, but with more significant astrocytic, perivascular oedema and collapsed microvessel. However, the blood brain barrier still appeared intact with no leakage of the lanthanum nitrate into either the endothelial cell cytoplasm or perivascular space.

Discussion

An important observation of our study was the finding that the administration of LPS to sham-operated, HD and BDL rats resulted in marked brain water increases. The greatest increase in brain water was seen in the HD rats administered LPS; which occurred without any observed effect on the level of consciousness. Only the BDL animals administered LPS reached pre-coma stages at 3 hours after administration of LPS. This lack of correlation between increased brain water and preserved mental state, in all rats except the LPS-treated BDL rats, suggests that factors in addition to hyperammonaemia and brain swelling contribute to the effects on consciousness found in HE.

The histological analysis (Figure 8) confirms the data obtained from brain water measurements, which show significant worsening of brain oedema in all groups following administration of LPS (Figure 4). This increase in brain water was characterised by astrocytic oedema that is indistinguishable from the well documented brain oedema seen in hyperammonemic liver failure models.(321, 322) The ionic tracer lanthanum nitrate (MW 433), was used as a highly reproducible method for EM detection of blood brain barrier integrity to paracellular movement.(323) Retention of lanthanum within the vessel wall in all the groups clearly indicates that the anatomical integrity of the blood brain barrier is maintained. Our observation differs from earlier findings in the rat models of ALF where the blood-brain barrier was observed to be compromised.(322, 323) The discrepancies between these earlier observations and our study may suggest a specific phenomenon of cytotoxic oedema in cirrhotic rats compared with a combined cytotoxic and vasogenic oedema in ALF.

Astrocyte oedema in the non-cirrhotic, hyperammonemic (HD) rats was associated with an increase in glutamine, reduction in *myo*-inositol and a marked increase in

the glutamine/*myo*-inositol ratio in keeping with the ammonia-glutamine-brain water hypothesis. Of note, is the observation that the severity of astrocytic oedema seen in the saline-treated BDL rats was markedly less despite similar degrees of plasma and brain ammonia levels as seen with the LPS-administered BDL group likely related to decreased glutamine, *myo*-inositol and glutamine/*myo*-inositol ratio, though non-statistical. Our observation adds to the existing debate about the role of glutamine in the development of astrocytic oedema during hyperammonaemia. (324)

The mechanism of the observed astrocytic swelling in BDL rats is therefore unclear, and possibly regulated by the effects of inflammation. The increase in brain oedema following LPS administration in each of the groups does not directly correlate with plasma and brain ammonia levels or brain ammonia metabolism as there was no difference in the ammonia, glutamine or myo-inositol levels between the rats administered LPS and the respective control groups. It has been previously shown that administration of LPS resulted in a sustained increase in TNF-α and F_2-Isoprostane levels in the BDL rats 3 hours post-injection compared with *sham*-operated rats administered LPS.(306) In support of this argument, higher NO levels are observed in BDL, *sham*-operated and HD rats following administration of LPS. In order to explore whether this altered peripheral inflammatory response was reflected in the brain, plasma and brain proinflammatory cytokine levels were measured. Our results show that LPS administration not only results in an increase in systemic cytokine response but also increases brain tissue TNF-α confirming our previous observation that a systemic inflammatory response may initiate brain inflammation during liver failure despite retained anatomical barrier integrity.

The BDL rats administered LPS showed significantly higher levels of nitrosation of brain proteins compared with all other groups. Ammonia-induced nitrosation of

astrocytic proteins has been demonstrated in isolated astrocytes and astroglial tissue in brain sections of portacaval anastomosed rats,(325) but hyperammonaemia is unlikely to be the sole mechanism for protein nitration as the NT/T ratio was not elevated in the hyperammonemic saline-treated BDL and HD rats. Similarly, LPS itself can lead to nitrosation of proteins in the brain,(326) but the observation of only a minimal increase in the NT/T ratio in sham-operated and HD rats administered LPS also argues against this being the only mechanism for protein nitrosation. It is conceivable that hyperammonaemia and LPS together provide an environment for nitrosation of brain proteins. However, the observation that the NT/T ratio remained unchanged in the HD rats administered LPS argues against the above hypothesis. The significant elevation of plasma NT/T and TNF-α levels in the LPS-treated BDL rats, has lead us to hypothesise that the subliminal inflammation that is a feature of BDL(327) may 'prime' the animal to the effect of ongoing hyperammonaemia and subsequent endotoxaemic/inflammatory insult. This hypothesis is supported by the recent demonstration in astrocyte cell cultures that physiological levels of TNF-α seen in liver failure, induces tyrosine nitration.(328)

As discussed, the differential progression to pre-coma observed in the LPS-treated BDL rats appeared not to directly correlate with any alteration in plasma and brain ammonia, electrolytes, brain osmolytes glutamine and *myo*-inositol, or even the degree of astrocytic oedema; despite changes consistent with the water-glutamine-brain osmolyte hypothesis. These observations appear to dissociate extent of cerebral oedema to degree of coma. It is possible that in the BDL rats' hyperammonaemia in some way acts synergistically with the systemic inflammatory response to alter the level of consciousness. This concept fits with our recent observation in cirrhotic patients, where induction of hyperammonaemia resulted in

worsening of neuropsychological tests when the patients were infected and showed evidence of an inflammatory response.(170) Following treatment of infection, similarly induced hyperammonaemia did not result in an alteration of neuropsychological function, supporting the hypothesis that ammonia and inflammation may be synergistic.(170) Also, our suggestion of synergy between hyperammonaemia and inflammation is also supported by the recent observation that BDL rats that were fed a high ammonia diet (and which showed evidence of low level of inflammation), developed increases in brain water.(327) However, hyperammonaemia and inflammation were present in the LPS-treated HD rats without any observed change in conscious level. Given the extent of brain oedema observed in the HD rats, with or without administration of LPS, it is surprising to note that there was no impact on the level of consciousness. This would suggest that there is an as yet undetermined additional factor critical in the progression to coma in cirrhotic rats. On this background of cirrhosis, hyperammonaemia and superimposed inflammation may then facilitate nitrosation of brain proteins, which may be important to neurological function.

The LPS-administered BDL model used in the present study was developed to encapsulate the pathophysiologic features of acute-on-chronic liver failure, which is starting to be recognised as a distinct clinical and pathophysiological entity. Accordingly, chronic liver disease was induced by ligation of the bile duct, which induces secondary biliary cirrhosis, and the superimposed injury was provided by the administration of LPS. This model contains the essential clinical features of cirrhosis - encephalopathy and mild renal dysfunction with exaggerated inflammatory response. Clinically, the animals are haemodynamically stable and following LPS are observed to have the typical changes in blood pressure response during the 3-hour time frame of the study.(320) Blood electrolytes and osmolarity

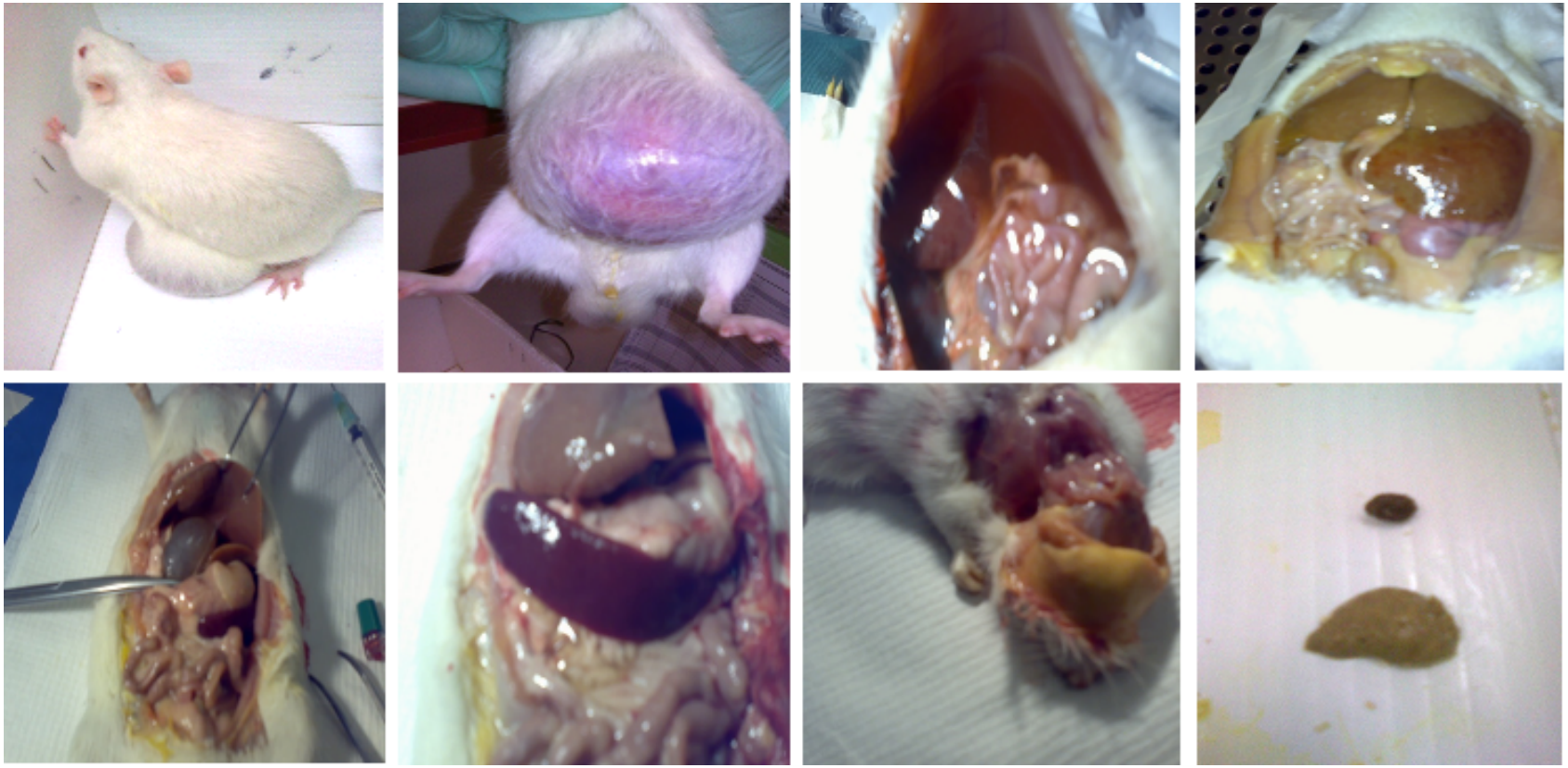
are maintained, reducing confounding effects. Whether the increase in brain oedema is associated with alterations in cerebral blood flow is not clear but the electron microscopic appearances of collapsed blood vessels possibly indicate reduced cerebral blood flow, which is in keeping with the observation of increased cerebrovascular resistance in humans with advanced cirrhosis.(329)

In conclusion, this study provides further evidence to indicate that on a background of cirrhosis and hyperammonaemia; superimposed inflammation has an important synergistic role in the development of HE. Further studies are needed to determine how ammonia and LPS facilitate cytotoxic oedema and pre-coma in cirrhosis and determine the role of nitrosation of brain proteins.

Legends to figures

Where symbols have been used, they represent the following statistical significance between groups; - $^{*}p<0.05$, $^{**}p<0.01$ and $^{***}p<0.001$ compared to sham-operated rats; $^{\$}p<0.05$, $^{\$\$}p<0.01$ and $^{\$\$\$}p<0.010$: compared to respective saline control rat; $^{\#}p<0.05$, $^{\#\#}p<0.01$ and $^{\#\#\#}p<0.001$ compared to HD.

Figure 1: Production of Bile duct-ligated rat model of cirrhosis



Production of bile duct-ligated rat models is associated with the development with jaundice, dark urine and ascites Production of bile duct-ligated rat models with A) distended abdomen (gross ascites), B) Ascites and dark urine, C) Ascites, D) Cirrhotic liver, E) Distended gall-bladder F) Splenomegaly, G) Jaundice and H) Pale stools (compared to a normal pellet stool with sham-operation

Figure 2: **Schematic of apparatus used with the Gravimetric technique**

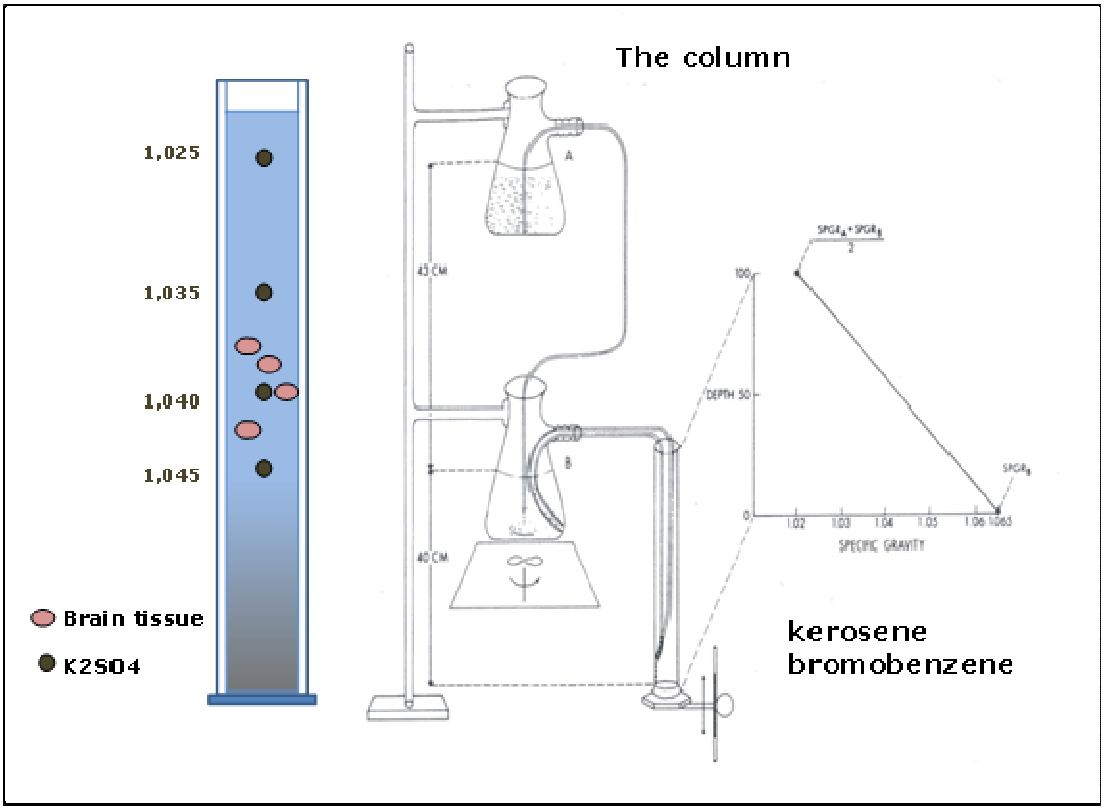
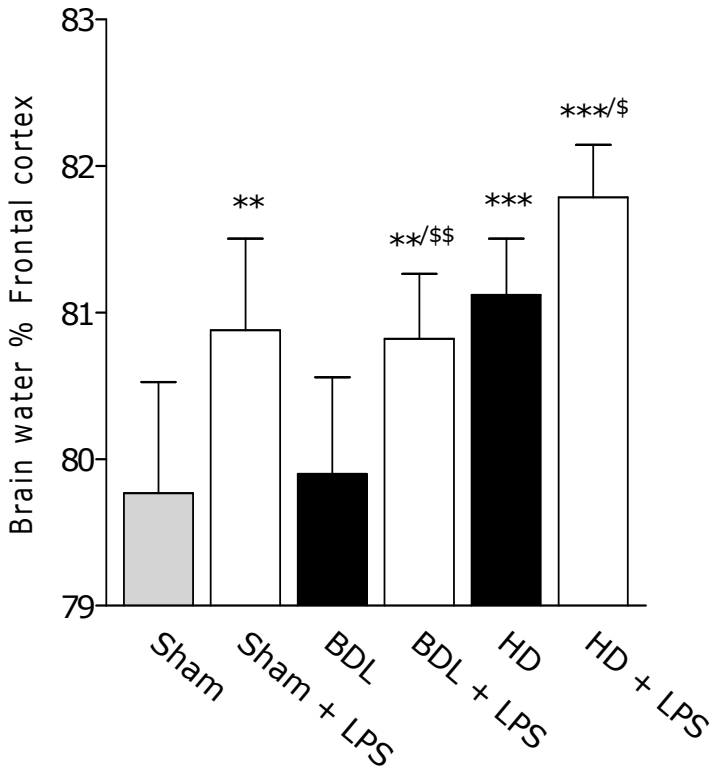


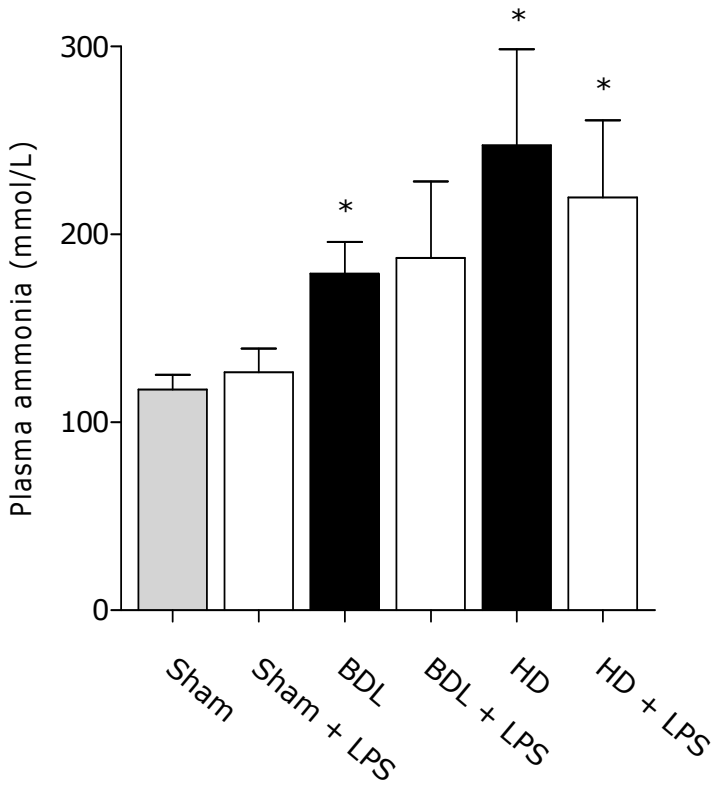
Figure 3: Perfusion & fixation apparatus

Figure 4: Frontal cortex brain water content



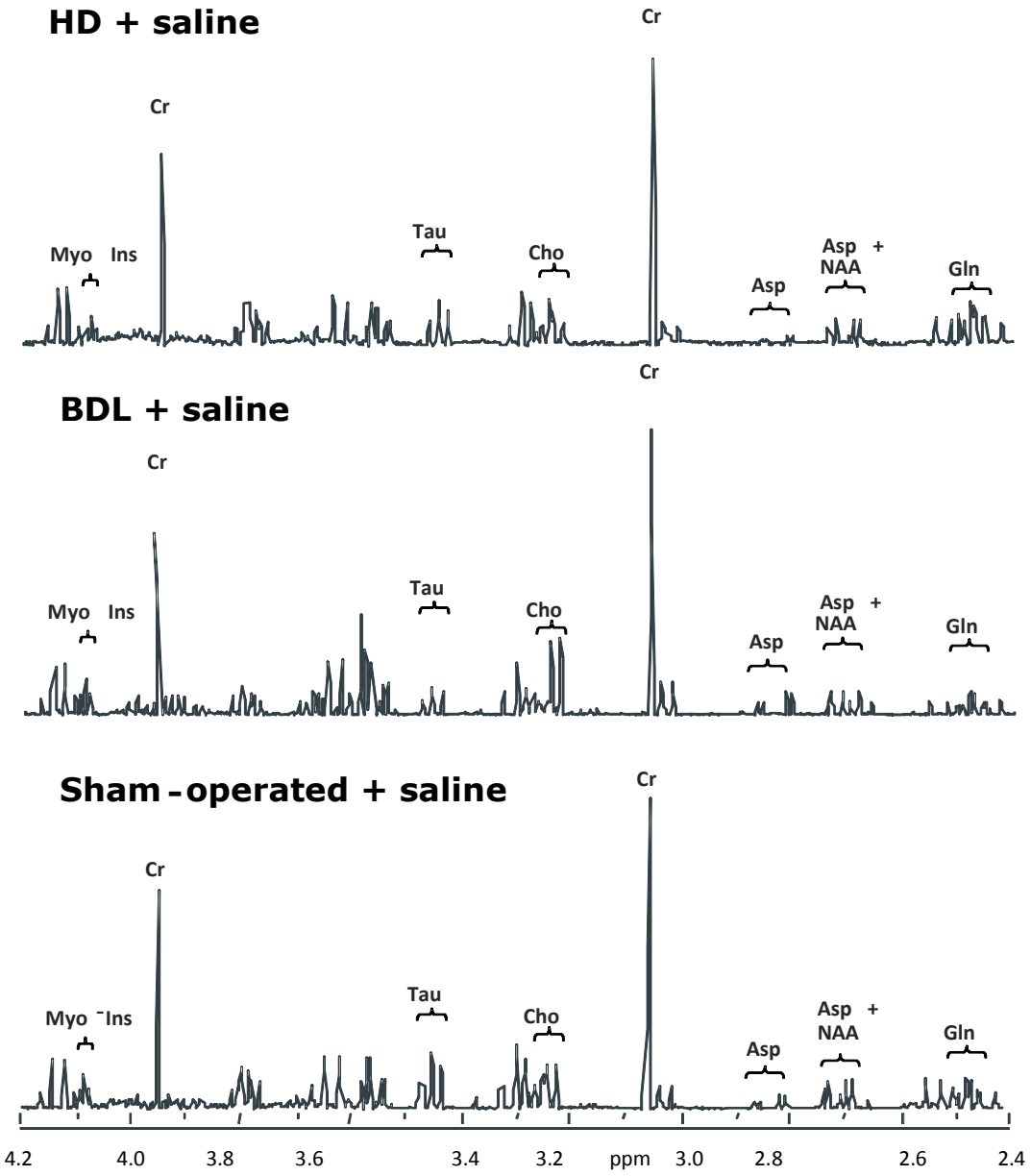
Compared to sham-operated rats, there was a significant increase in percentage frontal brain water content in HD ($p<0.01^{\$\$}$) and HD + LPS ($p<0.01^{\$}$) rats. There was no significant difference found between sham-operated and BDL saline-treated groups (p=0.237). Following LPS administration, there was an increase in brain water in sham-operated ($p<0.01^{**}$), BDL ($p<0.01^{\$\$}$), and HD ($p<0.01^{\$}$) rats. Significance was calculated using the Newman-Keuls Multiple Comparison Test (to determine differences between all groups).

Figure 5: Plasma ammonia levels



Plasma ammonia levels as determined by the indophenol detection method. Compared to sham-operated saline control rats, there was significantly elevated arterial ammonia in BDL ($p=0.019^{*}$), HD ($p=0.049^{*}$) and HD + LPS challenged ($p=0.042^{*}$) rats. There was no significant difference found between the BDL and HD saline-treated groups ($p=0.237$). Significance between two groups was established using T-test analysis.

Figure 6: ^{1}H-NMR spectra of brain extracts



Segments of ^{1}H-NMR spectra of brain extracts obtained from a sham-operated control, and from rats with BDL and HD. *Peak assignment abbreviations: Asp: aspartate; Cho: choline-containing compounds; Cr: creatine; Gln: glutamine; myo-Ins: myo-inositol; NAA: N-acetyl-aspartate; Tau: taurine.*

Figures 7a-c

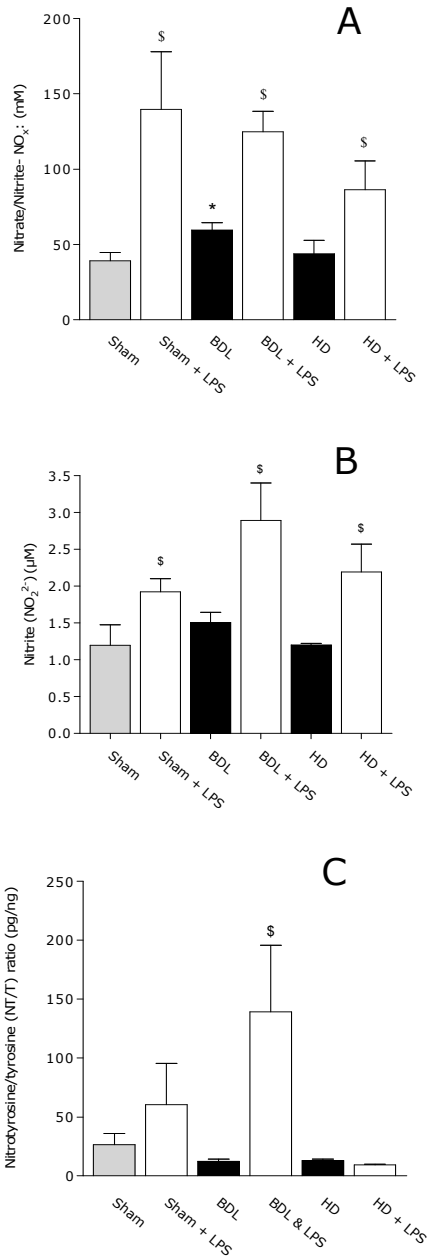


Fig A. **Plasma nitrite/nitrate levels:** There was a significant increase in plasma nitrite/nitrate levels with administration of LPS in BDL ($p<0.05^{\$}$), sham-operated ($p<0.05^{\$}$) and HD ($p<0.05^{\$}$) rats compared to the respective saline-treated controls; as determined using the T-test to compare 2 groups. A significant difference was also found between BDL + saline versus sham-operated + saline rats

($p<0.05$*). ***B.*** **Plasma nitrite levels:** There was a significant increase in plasma nitrite levels with administration of LPS in BDL ($p<0.05^{\$}$), sham-operated ($p<0.05^{\$}$) and HD ($p<0.05^{\$}$) rats; as determined using the T-test to compare 2 groups. ***C.*** **Brain nitrotyrosine levels:** There was a significant increase in brain nitrotyrosine level in LPS-treated BDL rats compared to saline-treated BDL rats ($p=0.01^{\$\$}$), as determined using the T-test to compare 2 groups.

Figure 8

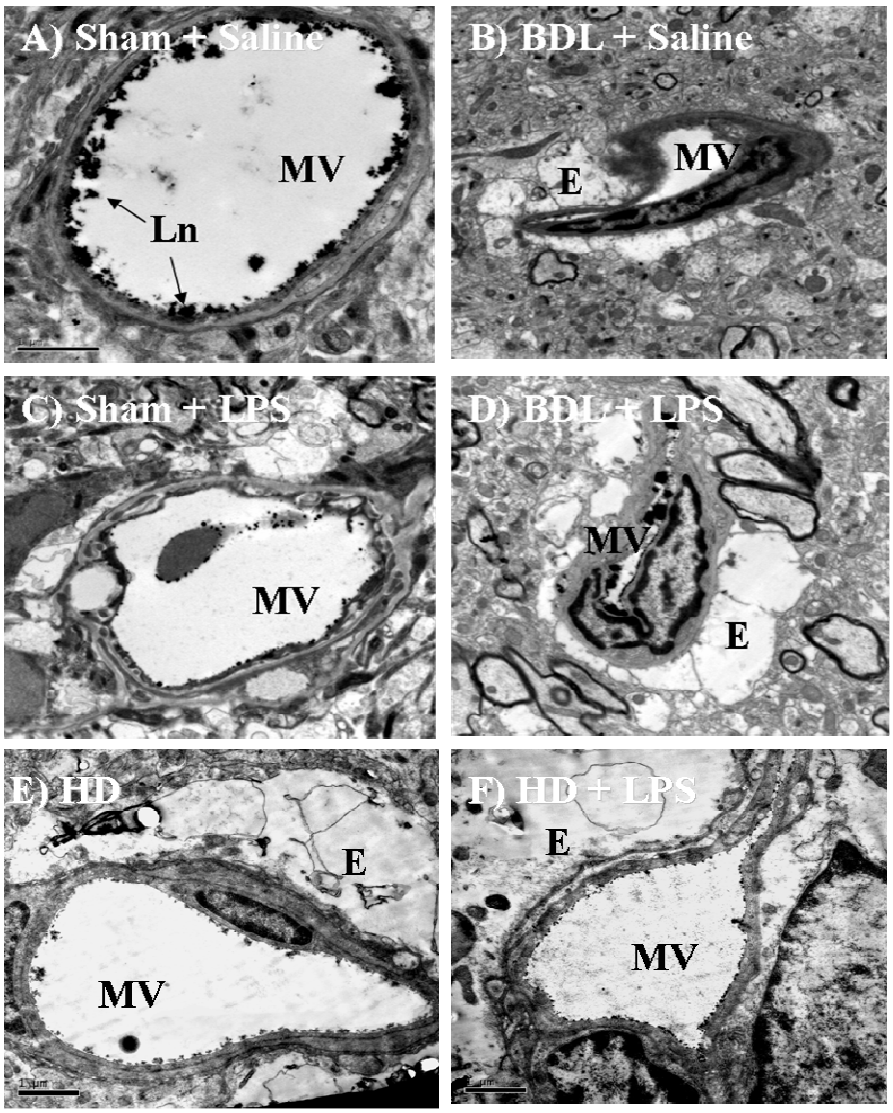


Figure 8 A-F: Brain histology: Transitional Electron micrographs (*Scale bar*, 1 μm), of frontal cortical brain sections from, **A)** ***Sham-operated rat***, showing normal microvessel architecture and lanthanum retained within the microvessel lumen; **B)** ***BDL rat***, showing a collapsed microvessel with moderate perimicrovessel oedema and lanthanum retained within the microvessel lumen; **C)** ***LPS-treated sham-operated rat,*** showing early perimicrovessel oedema and lanthanum retained within the microvessel lumen; **D)** ***LPS-treated BDL rat,*** showing a significantly collapsed microvessel with severe

perimicrovessel oedema, yet lanthanum still retained within the microvessel lumen; **E) *HD rat*** showing a partially collapsed microvessel, with intact endothelium shown by the retention of the lanthanum particles in the lumen; pronounced perivascular oedema can be seen. **F) *HD + LPS-treated rats***, showing similar disruption of structure to that found in BDL + LPS and HD rats, but with greater perivascular oedema and partial microvessel collapse. The blood brain barrier appears intact with no leakage of the lanthanum nitrate from the vessel lumen. *Abbreviations; (MV) = Microvessel, (E) = Perimicrovessel oedema* and *(Ln)* = the ionic tracer 'lanthanum nitrate' which is be visualised as small black densities opposing the luminal surface of the microvessel.

Table 1: Brain water, plasma ammonia, and brain nitrate, nitrite and nitrated proteins

	Sham	Sham + LPS	BDL	BDL + LPS	HD	HD + LPS
Frontal brain water (%)	79.7 ± 0.3	80.9 ± 0.2**	79.9 ± 0.3	80.8 ± 0.2**/$$	81.1 ± 0.1***	81.8 ± 0.2***/$
Plasma ammonia (µmol/L)	134 ± 12	127 ± 13	168 ± 14*	172 ± 37*	231 ± 47*	180 ± 38*
Nitrite/Nitrate	39 ± 6	139.7 ± 38*	59 ± 5*	125 ± 14*/$	44 ± 9	86 ± 19$
Nitrite	1.2 ± 0.3	1.9 ± 0.2*	1.5 ± 0.1	2.9 ± 0.5*/$	1.2 ± 0.0	2.2 ± 0.4*/$
NT/T	26 ± 10	60 ± 35*	12 ± 2	139 ± 56*/$	13 ± 1	9 ± 1

Values are given as mean ± standard error of mean (SEM) of duplicate samples from at least (n=6) rats per group. Statistical analysis was determined using the Mann-Whitney U test (for nitrite/nitrate studies) or an unpaired T-test (nitrite alone) to compare corresponding LPS and saline-treated groups. Inter group differences for brain water, NT/T ratios were calculated using the Newman-Keuls Multiple Comparison Test. Symbols represent;- *p<0.05, **p<0.01 and ***p<0.001 compared to sham-operated rats; and $p<0.05, $$p<0.01 and $$$p<0.010: compared to respective saline control rat. *Abbreviations: BDL, bile duct ligation; HD, high ammonia diet; LPS, lipopolysaccharide.*

Table 2: Plasma biochemical measurements

	Sham	Sham + LPS	BDL	BDL + LPS	HD	HD + LPS
ALT (U/L)	70 ± 6	90 ± 12	98 ± 15	168 ± 54*	31 ± 2	37 ± 4
Bilirubin (µmol/L)	10 ± 3	24 ± 6	180 ± 18*	181 ± 14*	28 ± 3	32 ± 3
Creatinine (µmol/L)	22 ± 1	24 ± 3	18 ± 0.0	27 ± 5	39 ± 2	32 ± 2
Urea (mmol/L)	5 ± 0.2	5 ± 0.6	5 ± 0.6	9 ± 0.4*$	7 ± 0.6	6 ± 0.9
Albumin (g/L)	35 ± 2	38 ± 1	26 ± 1*	24 ± 2*	39 ± 0.7	40 ± 0.7
Total protein (g/L)	46 ± 3	48 ± 2	54 ± 3	48 ± 3	51 ± 0.9	51 ± 1
Sodium (mmol/L)	139 ± 2	133 ± 3	141 ± 3	141 ± 3	134 ± 5	141 ± 5
Potassium (mmol/L)	4 ± 0.2	4 ± 0.6	6 ± 0.5	6 ± 0.5	5 ± 0.4	5 ± 0.1
Chloride (mmol/L)	104 ± 1	97 ± 2	106 ± 2	105 ± 3	97 ± 0.9	99 ± 0.9
Osmolarity (mOsm/L)	283 ± 22	270 ± 22	280 ± 23	282 ± 23	280 ± 41	289 ± 40

Values are given as mean ± standard error of mean (SEM) of duplicate samples from at least (n=6) rats per group. Statistical analysis was determined using the Newman-Keuls Multiple Comparison Test. Symbols represent;- $^{*}p<0.05$, $^{**}p<0.01$ and $^{***}p<0.001$ compared to sham-operated rats; and $^{\$}p<0.05$, $^{\$\$}p<0.01$ and $^{\$\$\$}p<0.010$: compared to respective saline control rat. *Abbreviations: BDL, bile duct ligation; HD, high ammonia diet; LPS, lipopolysaccharide.*

Table 3: Brain osmolyte profile as analysed by Proton NMR spectroscopy and brain ammonia

	Sham	Sham + LPS	BDL	BDL + LPS	HD	HD + LPS
Brain Ammonia (µmol/g ww)	0.27 ± 0.08	1.2 ± 0.17	1.0 ± 0.36	2.0 ± 0.66	0.61 ± 0.15	0.80 ± 0.26
Glutamine (µmol/g ww)	4.4 ± 0.21$^{\#}$	4.4 ± 0.12	3.5 ± 0.26$^{\#\#\#}$	3.8 ± 0.16	5.4 ± 0.31	5.6 ± 0.24
***Myo*-inositol (µmol/g ww)**	5.6 ± 0.31	5.6 ± 0.31	4.7 ± 0.28	5.5 ± 0.19	4.4 ± 0.23	4.8 ± 0.10
Creatine (µmol/g ww)	7.4 ± 0.11	7.3 ± 0.23	6.6 ± 0.32	6.9 ± 0.34	6.7 ± 0.21	7.5 ± 0.26
NAA (µmol/g ww)	4.1 ± 0.45$^{\#\#\#}$	4.1 ± 0.52	2.8 ± 0.14$^{\#\#\#}$	3.2 ± 0.13	6.1 ± 0.26	6.3 ± 0.26
Glutamine/*Myo*-inositol ratio	0.80 ± 0.06$^{\#\#\#}$	0.81 ± 0.06	0.73 ± 0.06$^{\#\#\#}$	0.69 ± 0.09	1.2 ± 0.09	1.2 ± 0.03

Values are given as mean ± standard error of mean (SEM) of duplicate samples from at least (n=6) rats per group. Where symbols have been used, they represent the following statistical significance between groups;- *p<0.05, **p<0.01 and ***p<0.001 compared to sham-operated rats; $^{\$}$p<0.05, $^{\$\$}$p<0.01 and $^{\$\$\$}$p<0.010: compared to respective saline control rat; $^{\#}$p<0.05, $^{\#\#}$p<0.01 and $^{\#\#\#}$p<0.001 compared to HD.

Table 4: Plasma and brain cytokine profile

	Sham	Sham + LPS	BDL	BDL + LPS	HD	HD + LPS
Plasma TNF-α (nmol/L)	0.03 ±0.02	0.19 ± 0.0	0.05 ± 0.02	1.7 ± 0.13$^{\#\#\#}$	0.02 ± 0.001	0.84 ± 0.40$^{\#}$
Plasma IL-6 (nmol/L)	0.053 ± 0.005	2.3 ± 0.27$^{\#\#\#}$	0.094 ± 0.035	2.1 ± 0.22$^{\#\#\#}$	0.077 ± 0.042	0.89 ± 0.42$^{\#}$
Brain TNF-α (nmol/L)	0.008 ± 0.001	0.065 ± 0.012	0.011 ± 0.002	0.055 ± 0.046	0.057 ± 0.019	0.060 ± 0.033
Brain IL-6 (nmol/L)	1.3 ± 0.24	1.4 ± 0.13	2.1 ± 0.20	2.0 ± 0.50	1.8 ± 0.14	2.0 ± 0.23

Values are given as mean ± standard error of mean (SEM) of duplicate samples from at least (n=6) rats per group. Statistical significance was calculated using Newman-Keuls Multiple Comparison Test. Where symbols have been used, they represent the following statistical significance between groups;- *p<0.05, **p<0.01 and ***p<0.001 compared to sham-operated rats; $^{\$}$p<0.05, $^{\$\$}$p<0.01 and $^{\$\$\$}$p<0.010: compared to respective saline control rat; $^{\#}$p<0.05, $^{\#\#}$p<0.01 and $^{\#\#\#}$p<0.001 compared to HD.

Research answers

1. Do systemic changes in ammonia and inflammation act synergistically to effect the progression and complications associated with HE as indicated by the depth of coma or cerebral oedema?

In the BDL model of cirrhosis the presence of hyperammonaemia and LPS acted synergistically to worsen brain oedema and coma.

2. With progression of HE, is there an anatomical or functional alteration of the blood brain barrier?

In the LPS challenged BDL model, there was no anatomical disruption of the BBB on electron-microscopic analysis.

3. On a background of hyperammonaemia and systemic inflammation, are the cerebral effects associated with HE due to alterations in brain ammonia and amino acid metabolism, and/or cerebral inflammatory responses?

In the LPS challenged BDL model, systemic inflammation caused a profound elevation of an already present low-grade proinflammatory state within the brain of BDL and to a lesser extent hyperammonemic rats.

4. Does the LPS challenged BDL rat model represent clinical of ACLF?

Characterisation of the LPS treated BDL model provides us with the first reproducible model representative of clinical ACLF and allows for further studies into the metabolic & cellular mechanisms involved with the pathogenesis of HE in ACLF?

Chapter 3

Hepatic Encephalopathy:

Interventional study -

Targeting Ammonia &

Amino Acid Metabolism

Published in '**Hepatology**' (2009)

Title:

"L-Ornithine and Phenylacetate Synergistically Produce Sustained Reduction in Ammonia and Brain Water in Cirrhotic Rats"

Authors:

Davies N. A, Wright G, Ytrebo L.M, Stadlbauer V, Fuskevag O.M, Zwingmann C, Davies D.C, Habtesion A, Hodges S.J and Jalan R

Abstract

Treatment of hyperammonaemia and hepatic encephalopathy in cirrhosis is an unmet clinical need. The aims of this study were to determine whether the combination, L-ornithine and phenylacetate (administered as the pro-drug phenylbutyrate) - 'OP', are synergistic and produce sustained reduction in ammonia, by L-ornithine acting as a substrate for glutamine synthesis thereby detoxifying ammonia and the phenylacetate excreting the ornithine-derived glutamine as phenylacetylglutamine in the urine. *Study 1:* Sprague-Dawley (SD) rats were studied 4 weeks after bile-duct ligation (BDL) or Sham-operation. Three hours before termination, an internal carotid sampling catheter was inserted and intraperitoneal (IP) – saline (placebo), OP (0.6g/kg), phenylbutyrate (0.6g/kg) or L-ornithine (0.6g/kg), were administered after randomisation. BDL was associated with significantly higher arterial ammonia and brain water, and lower brain *myo*-inositol ($p<0.001$, respectively) compared with sham-operation which was significantly improved in the OP treated animals; arterial ammonia ($p<0.05$), brain water ($p<0.05$), brain *myo*-inositol ($p<0.001$) and urinary phenylacetylglutamine ($p<0.01$). Individually, L-ornithine or phenylbutyrate were similar to the BDL group. *Study 2:* Four week BDL rats were randomised to saline or OP (IP) administered for 3, 5 or 10 days. Arterial ammonia and amino acids, brain water and brain osmolytes, and urinary phenylacetylglutamine were measured. The results showed that the administration of OP was associated with sustained reduction in arterial ammonia, brain water and markedly increased urinary excretion of phenylacetylglutamine. *Conclusion*: The results of this study provide proof of concept that L-ornithine and phenylbutyrate act synergistically to produce sustained improvement in arterial ammonia, its brain metabolism and brain water.

Background

Hepatic encephalopathy (HE) incorporates a spectrum of mental disturbances observed in patients with liver disease, ranging from minimal effects on quality of life, to coma and death.(6) Hyperammonaemia is considered central to the pathogenesis of HE, with arterial ammonia levels correlating with severity of intracranial hypertension and prediction of deaths from cerebral herniation in acute liver failure commensurate with an increase in brain delivery and uptake of ammonia.(116, 146) In cirrhosis, induction of hyperammonaemia has been shown to be associated with deterioration in neuropsychometric tests, worsening of brain osmolytes and increase in brain water.(307) The severity of HE in cirrhosis correlates with plasma ammonia levels confirming that ammonia lowering is an important goal for the treatment of HE. Therapy of hyperammonaemia and HE in patients with liver disease remains an unmet clinical need as the current ammonia-lowering therapies for HE have not been conclusively proven to be of value.(47, 48)

Studies of interorgan metabolism in liver failure have suggested that when the ammonia removing capacity of the liver is reduced, the muscles, gut and the kidneys interact to try and maintain ammonia levels.(112, 115, 133, 330) Glutamine acts as both a sink for excess ammonia, by ammonia combining with glutamate to produce glutamine (through the enzyme glutamine synthetase), or as a source for ammonia release (through the enzyme glutaminase).(61) Skeletal muscle glutamine synthetase activity is normally low, but has been shown to be up-regulated in liver failure.(131) Therefore, supplying glutamate to the large mass of skeletal muscle, increasing glutamine production, can be an alternative therapeutic target for ammonia detoxification.

As glutamate is not transported into muscle cells readily, glutamate can be provided to the muscle as L-ornithine, consuming ammonia to produce glutamine. This mode of delivery is utilised by the agent L-ornithine L-aspartate

(LOLA), which has been used for the treatment of the HE. However, although some randomised controlled trials with LOLA have reported reductions in plasma ammonia and improved psychometric test scores,(245, 248) recent observations suggest that the reduction in plasma ammonia with administration of LOLA maybe associated with a later increase in ammonia once LOLA is discontinued.(132) This is likely due to a significant rise in glutamine levels, which eventually becomes a source for ammoniagenesis by the kidney and gut through the effects of glutaminase.(133) This preliminary observation will have to be confirmed in future studies. Phenylacetate (administered as the pro-drug phenylbutyrate), which has been used to treat the hyperammonaemia of urea cycle enzyme deficiencies,(238) can effectively remove excess glutamine (derived from glutamate) to make phenylacetylglutamine which can be excreted by the kidneys, thereby removing it as a substrate for ammoniagenesis. However, unlike urea cycle enzyme deficiency where the liver is normal and usually the glutamine concentrations are markedly elevated; in cirrhosis the liver is dysfunctional and the plasma glutamine concentrations are variable being usually normal or mildly elevated;(115, 132, 133, 238, 307) making it unlikely that phenylacetate alone would be a useful treatment for HE.

Research Hypothesis

The above observations have led to the hypothesis for a novel ammonia-lowering therapy, combining L-ornithine and phenylacetate (OP),(132) for the treatment of hyperammonaemia and HE. OP has the advantage that any ammonia trapped as glutamine (derived from the administered L-ornithine) will not be available for later return to the circulation, resulting in net removal and reduction in ammonia concentration, Figure 1.

Research question

5. Can the novel therapeutic agent OP, by targeting inter-organ ammonia & amino acid metabolism, ameliorate the hyperammonaemia and development of advanced HE in cirrhotic rats?
6. What are the interorgan pathophysiological mechanisms involved?
7. Can ammonia lowering with OP last throughout a prolonged administration?
8. By lowering ammonia, can OP lead to a reduction in brain water?

Aim of study

The aims of this proof of concept study were to determine whether the combination, L-ornithine and phenylacetate are synergistic in reducing hyperammonaemia by L-ornithine acting as a substrate for glutamine synthesis thereby detoxifying ammonia and phenylacetate excreting the ornithine derived glutamine as phenylacetylglutamine in the urine. To test our hypothesis(132) we chose our well characterised bile duct ligated (BDL) rat model of cirrhosis as it exhibits characteristic hyperammonaemia, alterations in brain osmolytes and cytotoxic brain oedema.(331) The first part of the study was a 3 hour study to determine whether L-ornithine and phenylacetate were synergistic in reducing ammonia in this model compared with L-ornithine or phenylacetate alone. The second part of the study was designed to determine whether administration of OP achieved sustained reduction in ammonia over a 10 day period.

Declaration

This work was undertaken with a proportion of the funding from a Sheila Sherlock entry-level fellowship awarded by the European Association for the Study of Liver (EASL).

Methods

All animal experiments were conducted according to Home Office guidelines under the UK Animals in Scientific Procedures Act 1986. Male Sprague-Dawley (SD) rats (body weight 230–280g) were obtained from the comparative biological unit at University College London. All rats were housed in the unit and given free access to standard rodent chow and water, with a light/dark cycle of 12 hours, at a temperature of 19–23°C and humidity of approximately 50%.

Animal models

Bile-duct Ligation: Under anaesthesia (diazepam 1mg/kg intravenous (I.V), followed by Hypnorm® 150µl/kg intramuscular (I.M), Janssen Pharmaceutica, Belgium) all rats underwent bile duct ligation to induce biliary cirrhosis, or a Sham-operation as described previously.(306) In study 1, BDL rats were administered a high protein/ammoniagenic diet for 7 days prior to inclusion in the study. The diet consisted of a liquid rodent feed (Bioserve, Frenchtown, NJ 08825, USA) and a tailor-made mixture mimicking the amino-acid composition of haemoglobin molecule (4g/Kg/day Nutricia, Cuijk, The Netherlands) as described previously(115, 307) mixed with commercially available gelatin to prevent sedimentation. As described previously, this regimen produces chronic hyperammonaemia.(331)

Study Design

The following two studies were performed sequentially.

Study 1: Four weeks after surgery, the BDL animals were randomised into four groups. Three hours before termination, an intraperitoneal (IP) injection of, L-ornithine (0.6g/kg), phenylbutyrate (0.6g/kg), combined L-ornithine (0.6g/kg) and phenylbutyrate (0.6g/kg), or saline (placebo) were administered; doses based on preliminary dosing experiments. The sham animals were treated in the same manner but administered saline IP. The final study groups were: (1)

Sham-operated + saline (n=7) (2) BDL + saline (n=7), (3) BDL + ornithine (n=7), (4) BDL + phenylbutyrate (n=8) and (5) BDL + OP (n=8).

On the day of the experiment, following anaesthesia and immediately prior to the administration of study medication, a right internal carotid catheter (1.22mm portex fine bore polythene tubing, Scientific Laboratory supplies Ltd, Nottingham, UK) was inserted as previously described.(320) In brief, the procedure involved a midline cervical insertion with isolation and catheterisation of the right carotid artery (post-clamping). The catheter was held in place for the duration of the study by both a proximal and distal holding suture. Once in place blood sampling was undertaken. The catheter was kept patent by a heparinised saline block. The rats were allowed free access to food and water for the period of 3 hours post-intervention in a temperature controlled environment and were then sacrificed by exsanguination under anaesthesia (Hypnorm 200μL/kg I.M), 20 minutes after diazepam (1mg/kg I.P). Blood was withdrawn from the descending aorta and immediately put into ice cold heparin/EDTA containing tubes (until full exsanguination), centrifuged at 3120 x g and 4^{o}C, and the plasma collected and stored at -80°C until assayed. Urine samples were collected at the time of sacrifice by direct bladder puncture, and snap-frozen and also stored at -80°C. Brain samples, were also collected as described later.

Study 2: BDL animals were randomised into four groups. All animals were sacrificed at 4-5 weeks after the initial surgery. Study medications included IP injection of, L-ornithine (0.3g/kg), phenylbutyrate (0.3g/kg), combined L-ornithine (0.3g/kg) and phenylbutyrate (0.3g/kg), or saline (placebo) in two divided doses administered 10 hours apart. The sham animals were administered saline IP for 10 days prior to sacrifice. The final study groups were: (1) Sham-operated + saline (n=7) (2) BDL + saline (n=7), (3) BDL + OP for 3 days (n=6) (4) BDL + OP for 5 days (n=6) and (5) BDL + OP for 10 days (n=6). As per protocol, the rats sacrificed by exsanguination under anaesthesia

(Hypnorm 200μL/kg I.M), 20 minutes after diazepam (1mg/kg I.P). Blood, urine and brain samples were collected as described above.

Arterial ammonia, ornithine, glutamate and glutamine

250μL of plasma was deproteinised with 20mg of dry sulfosalicylic acid and the amino acid concentrations analysed by high performance liquid chromatography (HLPC) as previously described(332) (performed and analysed by Dr Nathan Davies, the Institute of Hepatology, UCL). Then 900μL plasma was deproteinised with 90μL trichloroacetic acid (TCA) for measurement of plasma ammonia concentrations. The substrate concentrations were measured by using a COBAS Mira S (Roche Diagnostica, Hoffman-La Roche, Switzerland).(333)

Brain water

Immediately after death, the whole brain was rapidly removed and 50mm^2 samples were dissected from the frontal cortex (grey matter). Brain tissue water content was determined using a previously described dry weight technique.(322, 334)

Brain Proton magnetic resonance spectroscopy (^{1}H-MRS)

Snap frozen cortical brain samples were processed and analysed by ^{1}H-NMR using a previously described technique(38) using the brain tissue from animals in Study 1 (performed and analysed by Dr Claudia Zwingmann, University of Montreal, Canada). ^{1}H-NMR spectra were recorded on a Bruker WB 360 spectrometer using a 5-mm QNP probe, 100-200 accumulations, repetition time 16 s, spectral width 3623 Hz, data size 16 K, zero filling to 32 K. Chemical shifts were referenced to lactate at 1.33 ppm. Total metabolite concentrations (μmol per gram wet weight) were analysed from ^{1}H-NMR spectra of PCA extracts using (trimethylsilyl)propionic-2,2,3,3d$_4$-acid as external standard.(314)

Urinary phenylacetylglutamine

The urine concentration of phenylacetylglutamine was measured using liquid chromatography tandem mass spectrometry (LCMS), based on a modified

method previously described(335) (performed and analysed by Dr L.M Ytrebo and O,M Fuskevag, University of Tromsø, Norway), In brief, the urine samples were prepared by adding 50µl of 0.1mol/L phosphate buffer solution (pH 2.4), 50µl of 60µmol/L internal standard, 3-(4-hydroxyphenyl)propionic acid (Sigma-Aldrich, Steinheim, Germany) and 50µl of urine in a 4.5 ml polypropylene tube (Sarstedt, Germany). To the same tube, 1ml of tert-buthyl methyl ether was added as the extractant. The tubes were capped, mixed by shaking for 1 min and centrifuged at 1700*g* for 180s. 750µl of the supernatant (in clean polypropylene tube) was evaporated to dryness under a stream of nitrogen at 40°C. The residue was then reconstituted in 100µl mobile phase. Samples were analysed by LC/MS/MS using Waters Acquity™ UPLC system with an auto-sampler and a binary solvent delivery system (Waters, Milford, MA) interfaced to Waters Micromass® Quattro Premier™ XE benchtop tandem quadrupole mass spectrometer (Waters, Manchester, UK). The chromatography was performed on a 100 x 2.1mm Waters Acquity™ BEH C_{18} 1.7mm column. The mobile phase consisted of 50% methanol in 10mmol/L aqueous formic acid with a flow rate of 0.2 ml/min (isocratic). The PAG standard was supplied by Bachem (Bubendorf, Switzerland). The method showed good linearity and reproducibility with a correlation coefficient (*r*) of >0.99 and a coefficient of variation of <5%.

Statistics

Data are expressed as mean ± SEM. Significance of difference was tested with Newman-Keuls multiple comparison test or two-way ANOVA; $p<0.05$ was taken to be statistically significant. Software used included Microsoft Excel 2003 (Microsoft Corp., Redmond, WA) and GraphPad Prism 4.0 (GraphPad Software, Inc., San Diego, CA).

Results

All rats continued to gain weight following surgery. From the final weight taken immediately prior to termination, BDL rats (mean ± SEM; 320g ± 14), were not significantly different to Sham-operated controls (mean ± SEM; 301g ± 19). The systemic hemodynamics in the BDL animals were well maintained as previously shown.(320, 331) All rats were alive after injection of study medication in each of the study groups.

Arterial ammonia

Study 1: Three hours after the administration of OP (0.6 mg/kg), the arterial ammonia levels were significantly reduced by 45% from the baseline value, from a mean ± SEM of 231.9 ± 18.7 to 150.7 ± 10.00 ($p<0.05$) (Figure 2A). The ammonia level was already reduced at 1 hour following administration of OP but did not reach statistical significance at this time (Table 1). The arterial ammonia levels achieved at the end of the 3 hour period in the OP animals was not significantly different to the sham animals. No significant change in ammonia was observed in the animals treated with placebo, L-ornithine (0.6 mg/kg) or phenylbutyrate (0.6 mg/kg) alone; Table 1 and Figure 2A.

Study 2: Bile duct-ligation induced significant hyperammonaemia ($p<0.001$) compared to Sham-operated controls (Figure 2B). Following administration of OP (0.6 mg/kg), there was a sustained a significant reduction in arterial ammonia at 3 ($p<0.001$), 5 and 10days ($p<0.01$, respectively) compared to BDL controls.

Ornithine, Glutamate and Glutamine

Study 1: There was a significant increase in the plasma ornithine concentration of the BDL rats administered L-ornithine ($p<0.05$) or OP ($p<0.05$), Table 1. This initial increase started to decline by 3 hours as the L-ornithine was metabolised. Following the increased circulating levels of ornithine and its later metabolism in the BDL + OP rats, an initial rise (at 1 hour) of glutamate was observed which then fell (at 3 hours), associated with a progressive rise in glutamine (from 1

hour) reaching statistical significance by T= 3 hours ($p<0.05$), Table 1. No significant change in ornithine level was observed in the phenylacetate alone group but the glutamine level at 3 hours was lower but this did not reach statistical significance. The changes in glutamate concentrations were not statistically significant in any of the groups.

Study 2: There was a significant and sustained increase in the ornithine concentration in the entire OP treated animals compared with the placebo treated groups. Ornithine concentration was markedly higher in the animals treated for 5 and 10 days compared with the animals treated for 3 days (Table 2). Similarly, glutamine concentrations were significantly higher in the OP treated animals reaching twice the concentrations observed in the placebo treated group. However, this was not significantly different between the animals treated for 3 days compared with the animals treated for 5 or 10-day periods. The change in glutamate concentration was not significantly different between any of the OP treated groups compared with the placebo treated group.

Brain water

Study 1: There was a significant increase in the water content in the frontal cortex of saline (and ammoniagenic feed) treated BDL rats compared with the corresponding Sham-operated rats, $p<0.01$; Figure 3A. This increase in brain water in BDL rats was significantly attenuated by administration of OP, compared with the BDL animals administered placebo ($p<0.05$) to values that were not significantly different to the Sham-operated controls. Brain water was not significantly different in the BDL rats given L-ornithine, or phenylbutyrate alone compared with the animals treated with placebo.

Study 2: With administration of OP, over 3, 5 and 10 days, there was a marked and significant reduction in frontal brain water content in each of the groups ($p<0.01$, respectively) when compared with the placebo treated animals which have a higher brain water content compared with the sham animals ($p<0.01$)

(Figure 3B). The brain water content in each of the OP treated groups was not significantly different to the sham group.

Proton NMR spectroscopy

Study 1. In BDL animals, brain glutamine was not different but the brain *myo*-inositol was lower ($p<0.001$, Figure 4), compared with Sham-operated controls, Table 2. Following administration of OP to BDL rats, there was a significant increase in brain myo-inositol levels ($p<0.001$, Figure 4). This resulted in a significant reduction in the glutamine/*myo*-inositol ratio ($p<0.001$) to values that were not significantly different to sham-operated controls. Following administration of L-ornithine or phenylbutyrate alone, there was no significant change in glutamine or myo-inositol levels, Figure 4 and Table 3.

Urinary phenylacetylglutamine

Study 1: In the sham group and the BDL animals treated with L-ornithine alone phenylacetate alone, the urinary concentration of phenylacetylglutamine was not significantly different to zero. There was a significant increase in urinary phenylacetylglutamine excretion following administration of OP to BDL rats compared to placebo treated BDL and Sham-operated control rats, $p<0.01$ respectively; Figure 5A.

Study 2: With administration of OP, over 6 hours, 3, 5 and 10 days, there was a significant increase in urinary phenylacetylglutamine excretion that increased with ongoing administration in each of the groups over the 10 day period ($p<0.01$, respectively) when compared to the sham animals and the placebo treated BDL control rats in whom the excretion of phenylacetylglutamine was not significantly different to zero (Figure 5B). The concentration of phenylacetylglutamine in the urine in the animals treated for 3, 5 and 10 days, was about 20 times higher in the urine compared with that observed at the 3 hour time-point. In the acute study (Study 1), the concentration of phenylacetylglutamine at the 3 hours time point was approximately

10micromol/L which was increased to approximately 200micromol/L in the animals treated for 3, 5 and 10 days.

Discussion

The results of this study demonstrates a synergy between L-ornithine and phenylacetate in reducing arterial ammonia levels in BDL rats to values that were not significantly different to sham operated controls. This reduction in ammonia was associated with a significant reduction in brain glutamine/*myo*-inositol ratio, which was associated with a normalisation of brain water. These effects of OP were sustained during a 10-day period of administration with respect to both a marked reduction in ammonia and also in brain water without any evidence of a rebound increase in ammonia. According to the proposed hypothesis, the reduction in ammonia was associated with an increase in arterial glutamine and urinary phenylacetylglutamine.

The observed reduction in ammonia in the OP treated animals was associated with an initial increase in circulating ornithine at 1 hour in both the OP and L-ornithine treated rats, which at 3 hours was substantially reduced. In accordance with the first part of the hypothesis, an increase in the glutamine at 3 hours was also observed. These changes in glutamine levels are consistent with previous observations of increased skeletal muscle glutamine synthetase activity and the effect of administration of LOLA to devascularised rats.(249) In the BDL animals treated with OP for up to 5 and 10 days, the ornithine levels continued to increase to twice the values seen in the animals treated for up to 3 days but the glutamine levels remained similar in the animals treated for 6 hours or 3 days compared with the animals treated for 5 or 10 day period indicating perhaps that the doses of ornithine used in OP could be reduced. This will however need to be tested in future studies.

The demonstration of phenylacetylglutamine in the urine of animals treated with OP supports the hypothesis that glutamine generated by the administration of L-ornithine can be conjugated and excreted as phenylacetylglutamine by co-administration of phenylbutyrate. It is interesting to note that the urinary

concentration of phenylacetylglutamine in the phenylacetate alone group was not significantly different to zero indicating possibly that the conjugation may require an elevated level of glutamine. This hypothesis will have to be tested in future studies. However, in the acute study (Study 1) there was a lack of stoichiometry between the reduction in arterial ammonia concentration and the increase in urinary phenylacetylglutamine. Although, this may be due to differences in conjugation pathways, as in humans, phenylacetylglutamine is the overwhelming renal excretion product of the reaction between phenylbutyrate and glutamine but in rats this association is less clear, as there are a number of additional by-products of phenylacetate metabolism.(336) It is more likely that the explanation may be related to kinetics of phenylacetylglutamine excretion. Phenylacetylglutamine is conjugated intracellularly and therefore requires time to be excreted from the system as illustrated by a 20-fold increase in the concentration of urinary phenylacetylglutamine in the animals treated for 3-10 days. The measured high concentration of phenylacetylglutamine in the urine together with twice the concentration of arterial glutamine in the OP treated animals explains in great part the observed reduction in ammonia. More detailed pharmacokinetic modelling studies will be necessary to determine exact relationship between the reduction in ammonia, urinary excretion of phenylacetylglutamine and the increase in glutamine concentration.

The results of ammonia reduction observed in the present study using OP has been confirmed in pigs with acute liver failure induced by hepatic devascularisation where over an 8 hour period the ammonia concentration was reduced by ~300μmol/L compared with a group treated with L-ornithine, phenylbutyrate or placebo indicating synergy between ornithine and phenylacetate.(337) Importantly, the effect was observed in animals that were effectively anhepatic. Similar increases in urinary phenylacetylglutamine were also demonstrated. Arterial ammonia was also shown to be reduced by about

30% over a 3 day period in 2 patients treated with a combination of L-ornithine and phenylacetate providing further proof for hypothesis.(132)

The most important finding of this study was the observation that the elevated brain water observed in the BDL animals was reduced in the OP group to values that were not significantly different to the sham operated controls. Again, this effect was maintained throughout the 10 day period of administration as observed with ammonia-lowering. Although there was an unexpected increase in brain water with the administration of phenylbutyrate and L-ornithine alone, this was not statistically significant when compared to placebo treated BDL controls. The effect of OP on the brain water in BDL animals is consistent with the observations in the porcine acute liver failure model where administration of OP prevented the rise in intracranial pressure to levels that were not significantly to sham operated controls.(337) The reduction in arterial ammonia following OP in the 2 patients referred to above was associated with an improvement in the mental state,(132) an observation that will have to be tested in appropriate clinical trials.

Several lines of investigations indicate that the cytotoxic brain oedema observed in patients with hyperammonaemia is associated with alterations in the brain osmolytes, which is characterised by an increased brain glutamine and likely compensatory reduction in *myo*-inositol.(149, 305, 338) Accordingly, the glutamine/*myo*-inositol ratio was used as a measure of this association, which was significantly higher in the BDL animals compared with placebo treated BDL controls, with a non-statistical 25% increase in glutamine and reduced *myo*-inositol consistent with the hypothesis. Interestingly, the glutamine/*myo*-inositol ratio was reduced in the OP treated animals to levels that were not significantly different to Sham-operated controls; due largely to an increase in brain *myo*-inositol levels was observed.(305, 307, 339) Although the rise in *myo*-inositol wih OP treatment in BDL rats is hard to explain and possibly counterintuitative to

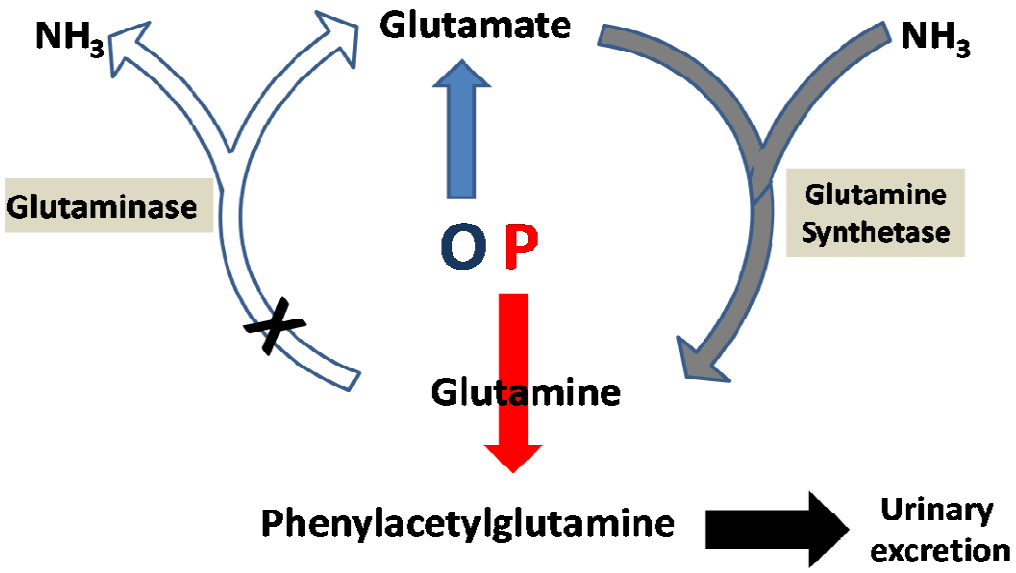
the 'ammmonia-glutamine-brain water hypothesis', it is possible that other intracellular osmolytes (e.g. taurine), or energy metabolsm or temporal considerations (e.g. lactate) are potential further important compensatory factors; this will require ongoing study.

In the groups treated with L-ornithine or phenylbutyrate alone, no significant changes in either glutamine or *myo*-inositol were observed. It is likely that the effect of OP on the brain osmolytes is due to a reduction in arterial ammonia, which was recently shown in devascularised pigs to be associated with a reduction in extracellular brain ammonia.(149) From the pathophysiological perspective, this prevention of reduction in *myo*-inositol may imply an increase in the brain buffering capacity to the effects of other precipitants such as a further hyperammonaemia or hyponatraemia; stimuli that are known to increase brain swelling.(33, 305) Similar corrections in brain osmolytes have been induced by treatment of clinically overt HE with Lactulose, which was principally shown to ameliorate reduction of brain *myo*-inositol with liver failure rather than correct the rise in intracellular glutamine.(340) Though the observed modulation of brain *myo*-inositol, which correlated with severity of HE, was observed after 1 week of lactulose, it is known that changes in levels of intracellular osmolytes like *myo*-inositol occur within hours,(341) as observed in this study. In rats given IP inositol, the levels of myo-inositol in the brain cortex rise by 33% as early as 2 hours.(341) This is likely to be associated with a very early and demonstrable increase in the transcription of brain Na^+/*myo*-inositol co-transporter (SMIT) in response to certain insults like changes of osmotic gradient.(342)

In conclusion, the results of this study provide support for our concept that combining L-ornithine with phenylbutyrate synergistically attenuates hyperammonaemia by increasing ammonia detoxification into glutamine and

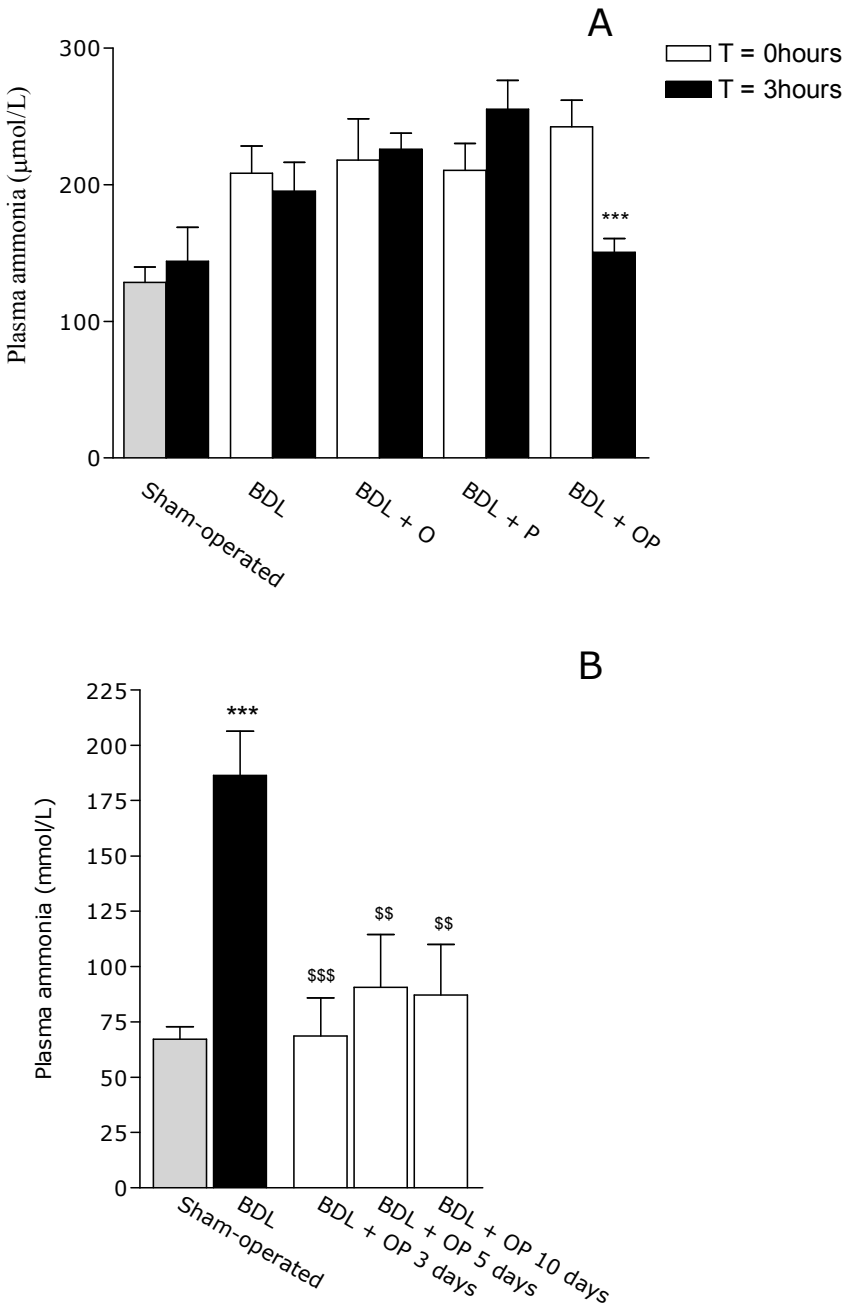
eliminating the resultant glutamine as urinary phenylacetylglutamine. Furthermore this ammonia-lowering effect is sustained over the 10-day period of administration without any evidence of a rebound phenomenon. We also show that OP by reducing plasma ammonia modulates brain osmolytes, which is associated with a reduction in brain water. The data indicate that OP is likely to be beneficial for the treatment of HE, and as the individual components, i.e. L-ornithine and phenylbutyrate are already in use in man, it is likely that these findings can be translated relatively quickly into appropriate clinical studies to determine exact dosing, pharmacokinetics, safety and efficacy.

Figure 1: Schematic demonstrating the glutamate-glutamine cycling and the hypothesised mechanism of action of the novel drug L-ornithine, phenylacetate (OP)



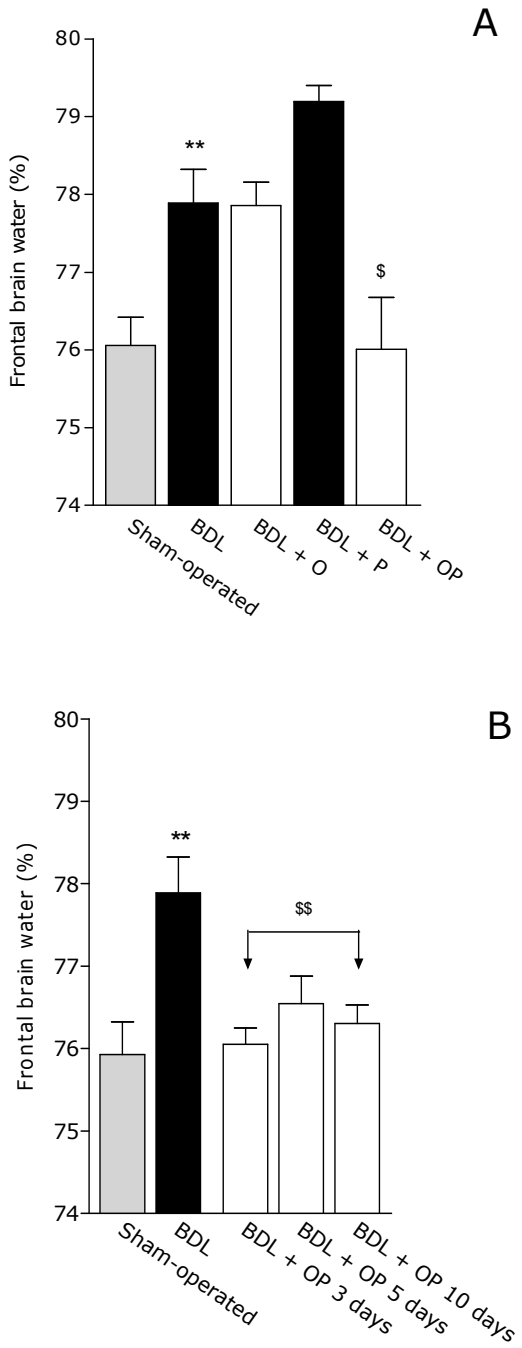
OP acts to reduce ammonia concentration in liver failure. L-ornithine acts as a substrate for glutamine synthetase, thereby detoxifying ammonia into glutamine. Phenylacetate combines with the glutamine that is generated, excreting it as phenylacetylglutamine.

Figure 2: Changes in arterial ammonia



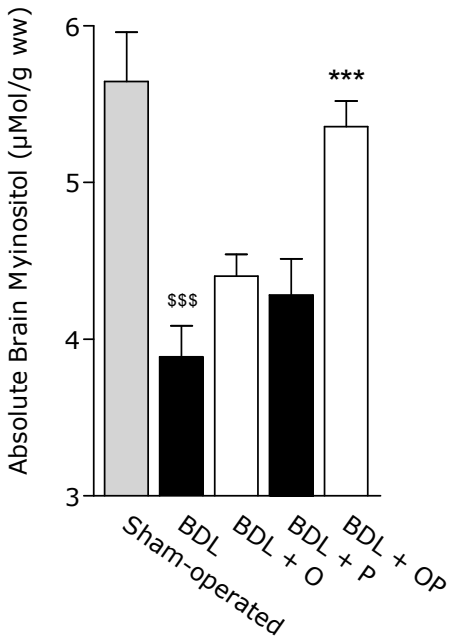
A) Shows a reduction in arterial ammonia concentration from baseline at 3 hours post administration of OP ($^{**}p<0.01$) compared to BDL controls, and at 3 hours were no different to Sham controls. **B)** Shows a sustained reduction in arterial ammonia following administration of OP for 3 ($^{\$\$\$}p<0.001$), 5 and 10days ($^{\$\$}p<0.01$, respectively) compared to hyperammonemic ($^{***}p<0.001$) BDL controls. *Abbreviations: BDL, bile duct ligation; OP, L-ornithine, phenylacetate; P, Phenylbutyrate; and O, L-ornithine.*

Figure 3: Frontal cortex brain water content:



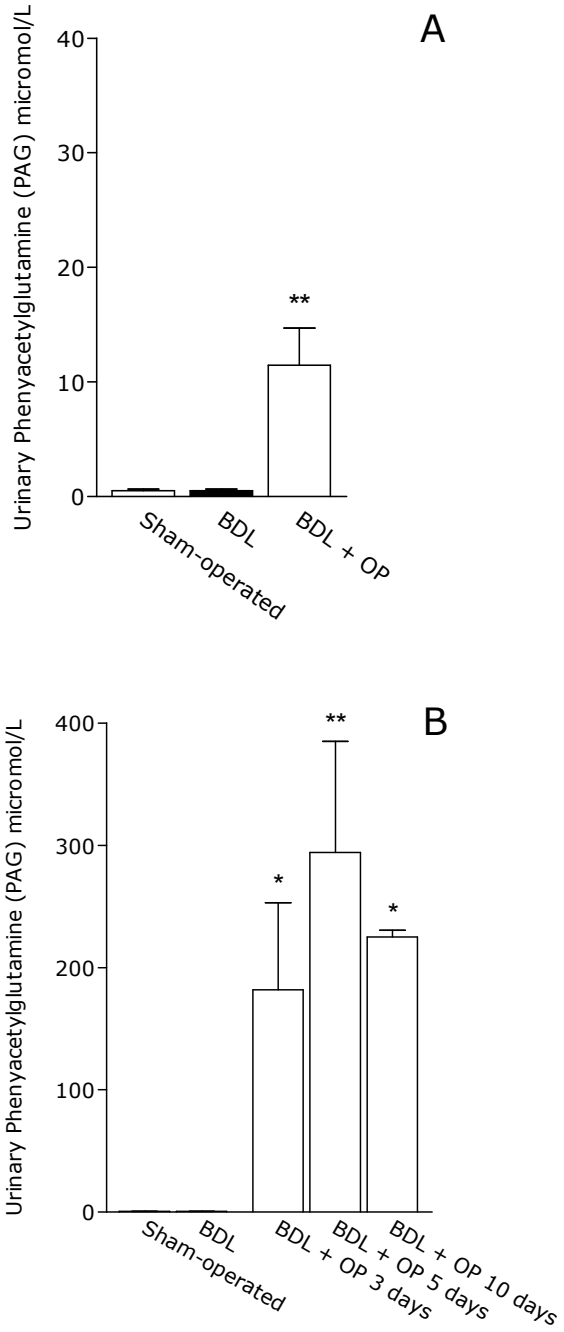
A) There was a significantly higher water content in the frontal cortex of BDL rats (**p<0.01) compared with Sham-operated controls. Administration of OP resulted in a significant reduction in brain water ($p<0.05) compared to BDL controls. **B)** Shows a sustained reduction in frontal brain water content (%) following 3, 5 and 10days ($$p<0.01, respectively) administration of OP compared to BDL controls with an established high water content (**p<0.01). *Abbreviations: Sham-operated, Sham-control; BDL, bile duct ligation; OP, phenylbutyrate; O, L-ornithine and P, phenylacetate.*

Figure 4: Absolute Brain *Myo*-inositol levels



Bile duct-ligation was associated with a significant reduction in brain *myo*-inositol level ($^{***}p<0.001$) Sham-operated controls. Administration of OP to BDL rats significantly increased the *myo*-inositol level ($^{\$\$\$}p<0.001$) compared to BDL controls, to levels that were no different to Sham-operated controls. Administration of L-ornithine or phenylbutyrate alone had no statistically significant effect on *myo*-inositol levels compared to BDL controls. *Abbreviations: Sham-operated, Sham-control; BDL, bile duct ligation; OP, phenylacetate; O, L-ornithine and P, phenylacetate.*

Figure 5: Urinary phenylacetylglutamine excretion



A) There was a significant increase in urinary phenylacetylglutamine following administration of OP to BDL rats ($^{**}p<0.01$) compared to both Sham-operated and BDL controls. **B)** Shows a profound and progressive increase in phenylacetylglutamine with OP administration over 3-10 days ($^{*}p<0.01$, respectively); peaking at 5 days ($^{**}p<0.001$) compared to BDL controls. *Abbreviations: Sham-operated, Sham-control; BDL, bile duct ligation; and OP, L-ornithine, phenylacetate.*

Table 1: Longitudinal changes (over 3 hours) in the plasma Ammonia, Ornithine, Glutamate and Glutamine

		Plasma ammonia and amino acid concentrations (µmol/L)			
	Hours	**BDL**	**BDL + O**	**BDL + P**	**BDL + OP**
AMMONIA	0	202 ± 20	218 ± 31	211 ± 20	232 ± 19
	1	211 ± 16	232 ± 44	300 ± 40	220 ± 24
	3	198 ± 24	217 ± 14	256 ± 21	151 ± 10*
ORNITHINE	0	51 ± 5	48 ± 4	46 ± 2	68 ± 14
	1	42 ± 2	448 ± 65*	49 ± 6	387 ± 35**
	3	66 ± 2	114 ± 20	45 ± 2	239 ± 82
GLUTAMATE	0	151 ± 7	256 ± 63	139 ± 11	135 ± 6
	1	126 ± 9	205 ± 74	207 ± 44	320 ± 55
	3	165 ± 19	172 ± 14	174 ± 30	212 ± 53
GLUTAMINE	0	605 ± 40	487 ± 62	633 ± 25	442 ± 62
	1	576 ± 25	663 ± 41	651 ± 35	663 ± 41
	3	670 ± 11	649 ± 52	516 ± 58	735 ± 67*

Data are expressed as mean ± standard error of mean (SEM) Symbols represent; - $^{*}p<0.05$, $^{**}p<0.001$ and $^{***}p<0.001$ compared to baseline (T= 0hours) for that group.

Abbreviations: BDL, bile duct ligation; OP, L-ornithine, phenylacetate; P, Phenylbutyrate; and O, L-ornithine.

Table 2: Longitudinal changes (over 10 days) in the plasma Ammonia, Ornithine, Glutamate and Glutamine

	Plasma ammonia and amino acid concentrations (µmol/L)				
	Sham-operated	**BDL**	**BDL + OP 3 days**	**BDL + OP 5 days**	**BDL + OP 10 days**
AMMONIA	67 ± 6	186 ± 20***	69 ± 17$^{\$\$\$}$	91 ± 24$^{\$\$}$	87 ± 23$^{\$\$}$
ORNITHINE	60 ± 35	51 ± 54	322 ± 43$^{\$\$}$	1293 ± 395$^{\$\$\$}$	1313 ± 279$^{\$\$\$}$
GLUTAMATE	101 ± 21	169 ± 21	256 ± 79	196 ± 27	238 ± 75
GLUTAMINE	490 ± 25	525 ± 36	1198 ± 291$^{\$\$}$	1297 ± 139$^{\$\$}$	1224 ± 143$^{\$\$}$

Data are expressed as mean ± standard error of mean (SEM) Symbols represent; - *p<0.05, **p<0.001 and ***p<0.001 compared to Sham-operated control rats; $^{\$}$p<0.05, $^{\$\$}$p<0.01 and $^{\$\$\$}$p<0.001 compared to BDL control rats. *Abbreviations: BDL, bile duct ligation; OP, L-ornithine, phenylacetate; P, Phenylbutyrate; and O, L-ornithine.*

Table 3: Brain osmolyte profile as analysed by Proton Magnetic Resonance Spectroscopy

	Sham-operated	BDL	BDL + O	BDL + P	BDL + OP
Glutamine (µmol/g ww)	4.4 ± 0.2	5.1 ± 0.2	4.8 ± 0.2	4.8 ± 0.2	5.1 ± 0.2
Myo-inositol (µmol/g ww)	5.7 ± 0.3	3.9 ± 0.2***	4.4. ± 0.1	4.3 ± 0.2	5.4 ± 0.2$$$
Creatine (µmol/g ww)	7.4 ± 0.1	6.6 ± 0.3	6.8 ± 0.3	6.8 ± 0.2	7.5 ± 0.2
Frontal brain water %	76.1 ± 0.4	77.9 ± 0.4*	77.9 ± 0.3$	79.2 ± 0.2$$$	76.0 ± 0.7$

Data are expressed as mean ± standard error of mean (SEM) Symbols represent; - *p<0.05, **p<0.001 and ***p<0.001 compared to Sham-operated control rats; $p<0.05, $$p<0.001 and $$$p<0.001 compared to BDL control rats. *Abbreviations: BDL, bile duct ligation; OP, L-ornithine, phenylacetate; P, Phenylbutyrate; and O, L-ornithine.* The brain osmolyte absolute values are expressed in micromoles per gram wet weight (µmol/g ww).

Research answer

1. Can the novel therapeutic agent OP, by targeting inter-organ ammonia & amino acid metabolism, ameliorate the hyperammonaemia and development of advanced HE in cirrhotic rats?

In a bile duct-ligated rate model of secondary cirrhosis, OP, due to the synergistic action of L-ornithine and phenylacetate, ameliorated hyperammonaemia.

2. Can any ammonia-lowering effect of OP last throughout a prolonged period of administration?

OP was able to maintain a reduction in plasma ammonia throughout a 10 day period of administration.

3. What are the inter-organ pathophysiological mechanisms involved?

As hypothesised, OP increases muscle glutamine production (through provision of L-ornithine) with its combination with phenylacetate and eventual excretion in urine as phenylacetylglutamine.

4. By lowering ammonia, can OP lead to a reduction in brain water?

There was an observed reduction in brain water with administration of OP.

5. What were the pathophysiological mechanisms involved?

Reduction in brain water was likely related to the restoration of brain *myo*-inositol levels driven by a reduction in extracellular brain ammonia induced by OP.

Chapter 4

Hepatic Encephalopathy:

Interventional study -

Targeting Ammonia & Inflammatory Pathways

Submitted to '**Hepatology**' (2009)

Title:

Reduction in arterial ammonia with L-ornithine Phenylacetate prevents LPS induced worsening in brain oedema and progression to pre-coma stages

Authors:

Wright G, Vairappan B, Stadlbauer V, Davies N.A, Hodges S.L and Jalan R

Abstract

In liver failure, inflammation synergistically modulates the cerebral effects of ammonia, with interventions targeting either hyperammonaemia or inflammation limiting progression of hepatic encephalopathy (HE). **Aim:** To test whether reduction in ammonia (with L-ornithine, phenylacetate - OP) prior to administration of LPS would prevent the deleterious brain consequence of LPS administration in a rat model of cirrhosis to mimic 'acute-on-chronic liver failure' (ACLF). Furthermore, to evaluate the role of the anti-TNF - Infliximab, on inflammatory pathways and in combination with OP. **Method:** Sprague-Dawley rats were studied 4 weeks post bile-duct ligation (BDL) or sham-operation (sham). BDL rats were randomised to 3 days intraperitoneal (IP) injections of OP (0.6g/kg, based on chapter 3 experiments) and/or Infliximab (10mg/kg, based on prior validated rat studies), or saline. Three hours before termination, all BDL rats received IP LPS (1mg/kg). Study groups were 1) sham 2) BDL + saline 3) BDL + LPS 4) BDL + LPS + OP, 5) BDL + LPS + Infliximab and 6) BDL + LPS + OP + Infliximab. Consciousness, brain water content, arterial ammonia, plasma biochemistry and regulatory (plasma and cortical brain) cytokines were assessed. **Results:** Survival analyses comparing BDL + saline, versus BDL + LPS rats was highly significant (Log-rank: $p<0.0001$), with OP significantly limiting progression to pre-coma/coma stages (Log-rank: $p<0.009$), but not with Infliximab. Compared to sham-operation, BDL was associated with significantly higher arterial ammonia ($p<0.001$), plasma TNF-α and IL-6 levels ($p<0.05$, respectively), but only a trend to increased brain water ($p=0.07$) and brain TNF-α and IL-6 levels. Addition of LPS significantly worsened coma stage, brain water ($p<0.01$) and augmented plasma TNF-α and IL-6 levels ($p<0.001$, respectively) and brain TNF-α levels ($p<0.05$), though non-significant increase in brain IL-6 levels. Giving OP to LPS-treated BDL rats significantly reduced arterial ammonia and brain water ($p<0.001$ and $p<0.01$, respectively), and lead to a non-

significant reduction in plasma and brain cytokines. Infliximab, significantly reduced plasma TNF-α and IL-6 ($p<0.001$ and $p<0.01$, respectively) with only a trend to reduced brain water ($p=0.167$) and brain TNF-α and IL-6 levels. Co-administered OP and Infliximab improved brain water (and coma score) but not significantly different to giving just OP alone ($p=0.3$). **Conclusion:** The findings of this study substantiate the predominant role of ammonia in priming the brain to the deleterious effect of LPS and suggest that a reduction in arterial ammonia concentration with OP may prevent LPS induced worsening of HE and brain oedema; though the mechanism of how ammonia primes the brain remains uncertain.

Background

Several studies support the hypothesis that ammonia and inflammation are synergistic in the pathogenesis of hepatic encephalopathy.(28, 174, 331, 343) This relationship is best described in patients with acute liver failure (ALF), where high arterial ammonia levels(21) and marked systemic inflammatory response(26, 28, 169) have independently been shown to result in severe HE. At advanced stages, increasing intracranial pressure (ICP) is associated with a marked proinflammatory response in the brain which directly correlates with cerebral blood flow(169) and an efflux (indicating production) of brain proinflammatory cytokines at terminal stages.(195) With its persistent low-grade endotoxaemia,(344, 345) cerebral oedema(3, 338) and insidious hyperammonaemia, this relationship is also evident with cirrhosis. In otherwise stable patients with minimal HE (MHE), systemic inflammatory responses modulate the neuropsychological manifestations of induced hyperammonaemia,(170, 174) with antimicrobials ameliorating HE progression.(170, 174) Additionally, cirrhotic patients often remain stable until infection, or high ammonia loads (e.g. gastrointestinal bleeding) trigger progressive encephalopathy indicative of acute-on-chronic liver failure (ACLF), a condition which can produce a clinical syndrome that mimics ALF.(7)

In bile duct-ligated (BDL) rat models of secondary biliary cirrhosis, there is a low-grade systemic and brain proinflammatory state indicated by elevated cytokines such as tumour necrosis factor-alpha (TNF-α))(327, 331) along with low-grade oedema.(331) In BDL rats, administration of LPS leads to a clinical situation that mimics clinical ACLF.(331) Our previous studies have shown that there is an exaggeration of both systemic and brain inflammatory response which results in worsening of the cytotoxic brain oedema resulting in a decline in consciousness to pre-coma/coma stages.(331) Experimentally, other groups have reported similar synergy with a recent study showing that treatment with

Minocycline reduces brain oedema in a devascularised animal model.(203) These data strongly support the hypothesis of a synergy between inflammation and ammonia in the pathogenesis of HE but how they interact to produce worsening of brain oedema is not clear.

In a recent study from the Copenhagen group, naive rats exposed to ammonia followed by intravenous bacterial lipopolysaccharide (LPS), were shown to develop an inflammatory response, cerebral vasodilation and intracranial hypertension,(343) which did not occur in animals administered LPS alone indicating the important role of ammonia in 'priming' the brain to the deleterious effect of LPS. It is well known that astrocytes are the key cells in the brain that are involved in HE and swell in response to hyperammonaemia according to the ammonia-glutamine-brain swelling hypothesis. As astrocyte foot processes also form part of the blood-brain barrier ammonia-induced swelling of the astrocyte may exaggerate brain swelling in response to a superimposed inflammatory stimulus.(346) The above discussion allows one to put forward the hypothesis that reduction in ammonia in cirrhotic rats would prevent LPS induced worsening of brain oedema and rapid progression to pre-coma and coma stages.

Recently, L-ornithine-phenylacetate (OP), a novel therapy targeting interorgan ammonia and amino acid metabolism,(132) has been shown to correct the hyperammonaemic state in animal models of cirrhosis(347) and ALF,(337) limiting brain oedema and rises in ICP. TNF-α is an important cytokine that has been implicated in the proinflammatory state associated with ALF and severe alcoholic hepatitis, with TNF-α deficient animals protected from brain oedema following induction of ALF.(348) Infliximab, a chimeric anti-TNF antibody is used clinically and has also been tested in alcoholic hepatitis patients(349, 350) and other especially rheumatological conditions.

Research hypothesis

1. Reducing circulating ammonia in cirrhotic rats would prevent LPS induced worsening of brain oedema and rapid progression to pre-coma and coma stages
2. Targeting both ammonia and inflammatory pathways will lead to a synergistic therapeutic benefit in the treatment of HE.

Research question

3. Can a reduction in ammonia in cirrhotic rats prevent LPS induced worsening of brain oedema and progression to pre-coma and coma stages?
4. Does targeting hyperammonaemia and inflammation together provide therapeutic synergy in the treatment HE?

Aim of study

To test whether reduction in ammonia with OP (prior to administration of LPS), prevents the deleterious brain consequence of LPS administration. Furthermore, the role of anti-TNF and combination therapy was investigated. Conscious level and brain water content acted as the primary end-points.

Methods

All animal experiments were conducted according to Home Office guidelines under the 'UK Animals in Scientific Procedures Act' 1986. Thirty-four male Sprague-Dawley rats, body weight 200–250g were obtained from the comparative biological unit at University College London. All rats were housed in the unit and given free access to standard rodent chow and water, with a light/dark cycle of 12 hours, at a temperature of 19–23°C and humidity of approximately 50%.

Animal models

Bile-duct Ligation: Under anesthesia - intravenous (IV) diazepam (1mg/kg), 20 minutes before intramuscular (IM) Hypnorm® (150µl/kg; Janssen Pharmaceutica, Belgium), all rats underwent bile duct-ligation (to induce secondary biliary cirrhosis), or sham-operation as described previously.(306)

Study design

Four weeks after surgery, BDL rats were randomised to receive 3 days of successive intraperitoneal (IP) injections of OP (0.6g/kg, based on chapter 3 experiments) and/or 10mg/kg (based on prior validated rat studies(351)) of reconstituted Infliximab (Schering-Plough UK), the mouse chimeric anti-TNF-α monoclonal antibody, or saline (placebo). Three hours before termination, all BDL rats were administered IP - 1mg/kg LPS (Sigma. Poole, UK). As controls, sham-operated rats only received IP saline. Study groups were 1) sham-operated, 2) BDL + saline, 3) BDL + LPS, 4) BDL + LPS + OP, 5) BDL + LPS + Infliximab, and 6) BDL + LPS + OP + Infliximab. The rats were allowed free access to food and water for the period of 3 hours post-intervention in a temperature controlled environment and were then sacrificed by exsanguination under anesthesia - IP Hypnorm® (200µL/kg), 20 minutes after IP diazepam (1mg/kg). Blood was withdrawn from the descending aorta and immediately, within seconds, put into ice cold heparin/EDTA containing tubes (until full

exsanguination), centrifuged at 3,120 x g and 4°C, and the plasma collected and stored at -80°C until assayed.

Assessment of level of consciousness

The conscious levels of rats used in this study were rated using an established neurological scale.(308) It was rated as either normal, with loss of the scatter reflex (c.f. Grade 1 encephalopathy) and ataxia (c.f. Grade 2 encephalopathy), together representing pre-coma stages, or loss of the righting reflex (c.f. Grade 3-4 encephalopathy) representing the coma stage.

Brain water

Immediately after death, the whole brain was rapidly removed and 50mm^2 samples were dissected from the frontal cortex (grey matter). Brain tissue water content was determined using a previously described dry weight technique.(322, 334) Briefly, oven dried Eppendorfs were weight (with a sensitive electronic scale), then 50mm^2 wet frontal cortex samples placed into each respectively labelled eppendorf and reweighed; all samples where within 0.1 mg difference. The dry weight was determined after Eppendorfs loaded with individual brain samples were spun in a vacuumed Eppendorf centrifuge at 10,000 x g and 40°C for 2 days. Tissue water content was then calculated as % H_2O = (1-dry wt/wet wt) x 100%

Plasma biochemistry and arterial ammonia

Substrate concentrations were measured using a COBAS Integra multiple analyser (Roche Diagnostics, UK) as previously described.(333)

Plasma and cortical brain cytokines

Plasma and cortical brain samples were snap frozen (-80°C) and stored. Prior to analysis, 100µg of cerebral cortex was homogenised and deproteinised (using a glass tube Teflon pestle homogeniser) in 300µl of ice-cold cell lysis buffer solution. After centrifugation at 12,000 x g for 10 minutes at 4°C, the supernatants were collected for processing. Following protein concentration

quantification of equilibrated brain protein samples and plasma supernatants (50µl) were analysed for cytokine levels (pg/ml) by flow cytometry using the Becton Dickinson (BD™ biosciences) rat inflammation cytometric bead array (CBA) kit as described by the manufacturer's instructions. These included the proinflammatory cytokines - TNF-α and interleukin-6 (IL-6). Samples were analysed by measuring the fluorescence produced by the CBA beads on a 'FACS Canto™ II flow cytometry system (BD™ Sciences) and the data analysed with BD™ CTA software.

Statistics

Data are expressed as mean ± SEM. Significance of difference was tested with Newman-Keuls multiple comparison test or two-way ANOVA; $p<0.05$ was taken to be statistically significant. Paired t test or Wilcoxon Signed Rank test was used for comparison of two groups as appropriate. Kaplan-Meier survival analyses were performed for the time to pre-coma/coma in the different treatment groups and the log-rank test was used for statistical analysis of the data comparing the survival curves. Software used included Microsoft Excel 2003 (Microsoft Corp., Redmond, WA) and GraphPad Prism 4.0 (GraphPad Software, Inc., San Diego, CA).

Brain proton magnetic resonance spectroscopy

^{1}H-MRS was not utilised in this study as changes in brain osmolytes with OP were assessed in the experiments undertaken in chapter 3.

Results

All rats continued to gain weight following surgery. From the final weight taken immediately prior to termination, BDL rats (mean ± SEM; 342g ± 42) were not significantly different to sham-operated controls (mean ± SEM; 380g ± 38). The systemic haemodynamics in the BDL animals were well maintained as previously shown.(320, 331) All rats were still alive 3 hours after injection of study medication.

Neurobehavioral changes

All rats were still alive 3 hours after injection of study medication, with sham-operated rats administered saline remaining fully alert throughout the 3 hour study period. All rats administered LPS displayed pilo-erection or hunched posture within 30 to 60 minutes. LPS treated BDL rats began to show a marked deterioration in conscious level, reaching pre-coma (n=2/6) and coma (n=4/6) stages by 3 hours. In LPS treated BDL rats administered lone OP, only (n=3/6 developed pre-coma/coma, while in LPS treated BDL rats administered lone Infliximab (n=5/6) developed pre-coma, with one progressing to coma (n=1/6). However, in LPS treated BDL rats co-administered OP + Infliximab, only (n=3/6) progressed to pre-coma/coma. Survival analyses comparing BDL + saline with BDL + LPS was highly significant (Log-rank: $p<0.0001$). Treatment of the BDL + LPS group with OP had significantly less progression to pre-coma/coma stages (Log-rank: $p<0.009$) which was not the case on treatment with Infliximab (Log-rank: $p=0.3$; Figure 1).

Brain water measurements

Though there was a limited increase in the brain water content of the frontal cortex between saline administered sham-operated and BDL control groups, this did not reach statistical significance (Figure 2 and Table 1). There was a significant increase in brain water in LPS challenged BDL rats compared with saline-administered sham-operated and BDL controls ($p<0.01$, respectively).

This increase in brain water with LPS in BDL rats was significantly attenuated by administration of OP ($p<0.01$) but not with lone administration of Infliximab ($p=0.167$). Co-administration of OP and Infliximab significantly reduced brain water ($p<0.05$) to levels that not significantly different to saline treated BDL controls. However, this reduction was not statistically different from the lone OP treated group ($p=0.3$).

Arterial ammonia

When compared to sham-operated controls bile duct-ligation lead to a significant increase in plasma ammonia ($p<0.001$; Figure 3 & Table 1). Following the administration of OP (± Infliximab), the arterial ammonia levels were significantly reduced ($p<0.001$, respectively) when compared to BDL (± LPS) rats. No significant change in ammonia was observed following the administration of either LPS or Infliximab ($p=0.5$ and $p=0.4$ respectively).

Plasma biochemistry

Compared to sham-operated rats there was a significant increase in bilirubin ($p<0.01$) consistent with secondary cirrhosis following bile duct-ligation (Table 1). In BDL rats, administration of LPS had no demonstrable effect on plasma biochemistry, with albumin, renal function, electrolytes and plasma osmolarity not statistically different between study groups.

Plasma and cortical brain cytokines

Throughout all study groups, the mean frontal cortical brain tissue cytokine levels were in the order of 10-fold higher compared to their respective circulating plasma levels. Multiple comparison group analysis revealed the following:

Plasma cytokines: When compared to sham-operated rats, bile duct-ligation was associated with a significant increase in the plasma levels of the proinflammatory cytokines TNF-α and IL-6 ($p<0.05$, respectively; Table 2). In LPS challenged BDL rats, TNF-α and IL-6 levels were significantly elevated when

compared to sham-operated controls ($p<0.001$, respectively) and saline-treated BDL controls ($p<0.01$, respectively). In LPS treated BDL rats, lone administration of Infliximab significantly ameliorated elevated TNF-α and IL-6 ($p<0.001$ and $p<0.01$, respectively). When compared to BDL + LPS rats, there was a significant reduction in plasma TNF-α ($p<0.01$) and non-significant trend towards reduction in IL-6 following lone administration of OP. In LPS treated BDL rats, co-administration of OP and Infliximab significantly ameliorated elevated TNF-α and IL-6 ($p<0.001$ and $p<0.01$, respectively), statistically no different to the effect with lone Infliximab.

Cortical brain cytokines: When compared to sham-operated rats, bile duct-ligation was associated with a marked but not significant increase in the brain levels of TNF-α and IL-6 ($p>0.05$, respectively; Table 2), which though augmented by LPS, still failed to reach significance. In LPS treated BDL rats, lone administration of Infliximab or OP, did ameliorate the elevated cortical brain tissue levels of TNF-α and IL-6 though this proved non-significant. Co-administration of OP and Infliximab lead to a greater reduction in TNF-α and IL-6, which only reached significance with IL-6 ($p=0.03$).

Discussion

The results of this study show for the first time that reduction in arterial ammonia concentration using OP in BDL rats is associated with a reduction in LPS induced progression to pre-coma/coma which is associated with a marked attenuation in brain swelling. These data provide a strong argument in support of the hypothesis that, on the background of chronic liver disease (BDL), ammonia may prime the brain to the deleterious effect of LPS. These data may have important therapeutic consequences. As clinically overt HE in cirrhosis is usually associated with a precipitating illness such as infection, these data provide support for the hypothesis that if ammonia levels could be kept low in patients with existing cirrhosis, sepsis induced occurrence of HE could be prevented.

The animal model chosen to explore this hypothesis, the LPS administered BDL rat, has been carefully characterised by our group and closely resembles the clinical situation of precipitated HE. BDL for 4-weeks prior to the study represents chronic liver disease with hyperammonaemia and a proinflammatory state (indicated by elevated arterial and brain cytokines). Additionally, the administration of LPS to this model is reflective of a second hit, and in this context represents an infective episode with evidence of exaggeration of the inflammatory response manifested by an increase in TNF–α and IL-6. I have previously shown by electron microscopy, that cirrhotic brain exhibits the classical 'cytotoxic oedema' even in the non-LPS treated group (chapter 2).(331) As ammonia and inflammation work simultaneously (and in synergy) to produce brain oedema and coma, this study used the BDL + LPS rat model to explore whether reduction in ammonia or inhibition of inflammation would prevent LPS induced worsening of brain oedema. For this study, OP was chosen to reduce arterial ammonia prior to LPS challenge following on from our recent observations in the BDL rat and devascularised pig models,(337, 347) which

confirmed that OP successfully reduces arterial ammonia concentration.(337, 347) In order to explore the effect of inhibiting the inflammatory response, Infliximab, a chimeric anti-TNF antibody that targets the regulatory pathways influenced by the proinflammatory cytokine TNF-α, was used. This strategy was chosen based upon our previous studies showing that TNF responses in BDL animals treated with LPS were exaggerated(306) and our observations in humans about the critical role of TNF–α in the cerebral hyperaemia and intracranial hypertension with ALF.(169) Infliximab was shown to reduce inflammatory responses in patients with severe alcoholic hepatitis with improvement in vascular function.(349, 350, 352) As predicted, administration of Infliximab was associated with marked attenuation of the proinflammatory drive manifested by a reduction in arterial TNF-α and IL-6 concentrations. Of particular interest was the observation that OP treatment was to some extent associated with a reduction in plasma and brain proinflammatory cytokines. This finding further ties plasma and brain ammonia and inflammatory responses to HE pathogenesis.

The most important observation of this study was the finding that reduction in ammonia with OP reduced LPS induced development of pre-coma/coma and worsening of brain oedema, which was not observed in the groups treated with Infliximab. It was therefore not surprising that co-administration of OP and Infliximab had similar effects to that observed in the OP treated group. These observations provide proof for the concept that ammonia may in some ways 'prime' the brain to the effects of LPS. This conclusion is further supported by our previous observation that the administration of LPS alone to naive rats did not result in pre-coma/coma stages (chapter 2).(331) The failure of Infliximab to exert a significant effect is unlikely to be due to the dosing and regimen used as Infliximab was observed to adequately reduce plasma and brain TNF-α levels.

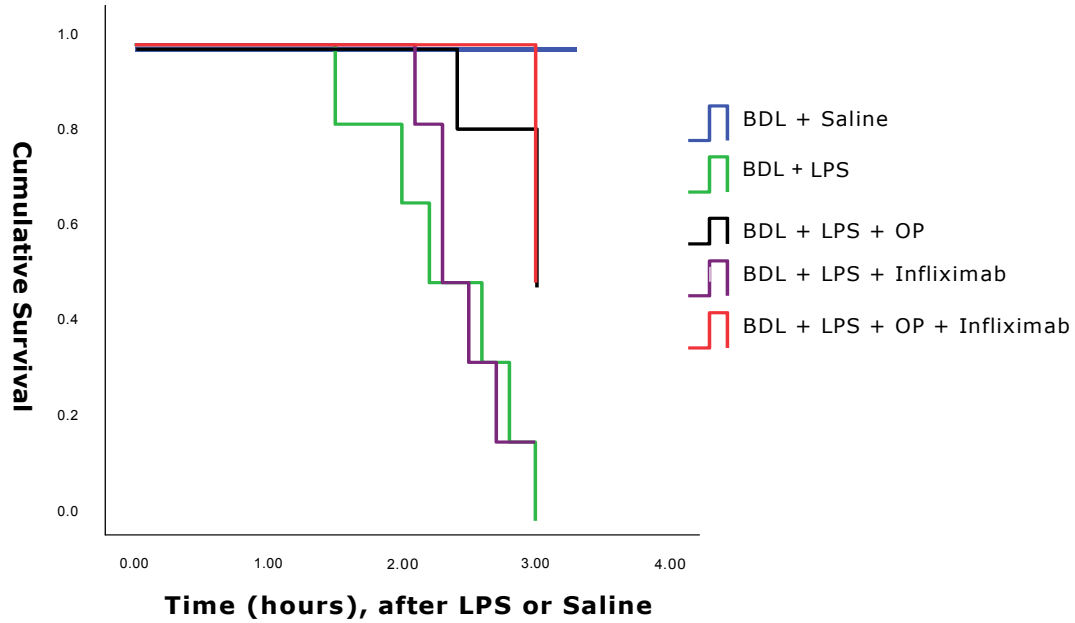
The mechanism of how ammonia may prime the brain to the effect of LPS is a matter of conjecture. Given the established central role of astrocytes (despite the possible impact of microglia(202, 203) and pericytes(207, 353)), recent reports of differential Toll-like receptors (TLRs) responses to microbial challenge on astroglial cells(354) may provide greater understanding of the interaction between inflammatory responses and ammonia(331, 355, 356) (e.g. neutrophil swelling and activation(356)); especially given any resultant influence on cerebral haemodynamics(201, 357, 358) and barrier integrity (whether mechanical or functional). TLRs are a class of receptors that recognise certain microbes (e.g. TLR-2 - lipopeptide on gram-positive bacteria and TLR-4 - LPS on gram-negative bacteria), stimulate proinflammatory cytokines and other mediators to activating innate (and adaptive) immunity. In the brain of BDL rats, our group have previously found up-regulation of TLR-2 and TLR-4, which was further augmented by LPS.(359) Our group has also demonstrated this pattern of TLR expression similarly influences neutrophil phagocytosis in patients with alcoholic hepatitis, triggered by humoral endotoxin.(355) Furthermore, we have also shown that administration of albumin was found to ameliorate TLR-2 and TLR–4 upregulation.(359) Such specific patterns of TLR upregulation may therefore prime the cirrhotic rat brain (and other organs(355, 359)) to further microbial challenge triggering a progression to ACLF.

Although it appears that reduction in ammonia is the more important factor in prevention of LPS induced deterioration in HE and brain oedema, several lines of investigation indicate that inflammation may well be a necessary target of future therapies. Interventions suppressing proinflammatory responses that have been shown to be clinically beneficial include hypothermia and the cyclooxygenase (inhibitor Indomethacin in acute liver failure,(146) albumin(140) and albumin dialysis(140) and reduced bacterial translocation with probiotics(220)).

However, modulation of inflammatory processes is difficult as interventions that targeting inflammation may predispose to increased risk of infection.

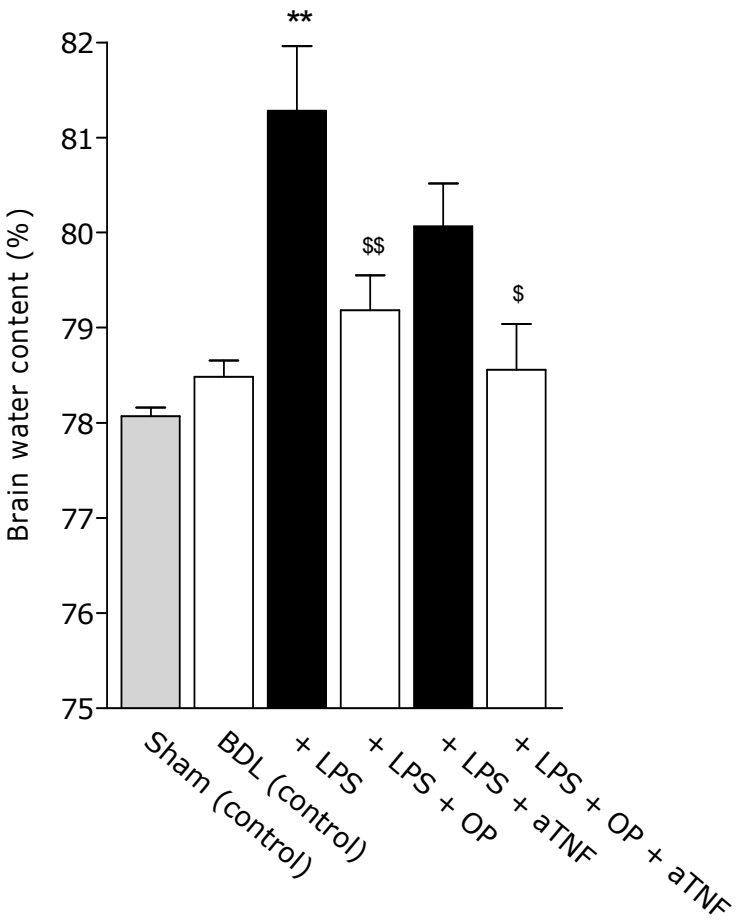
In conclusion, the findings of this study support a role for ammonia in priming the brain to the deleterious effect of LPS. Furthermore the findings suggest that a reduction in arterial ammonia concentration, possibly associated in a reduction in the proinflammatory state, with OP may prevent LPS induced worsening of HE and brain oedema. However, the mechanism of how ammonia primes the brain remains uncertain.

Figure 1. Time to pre-coma/coma with study intervention



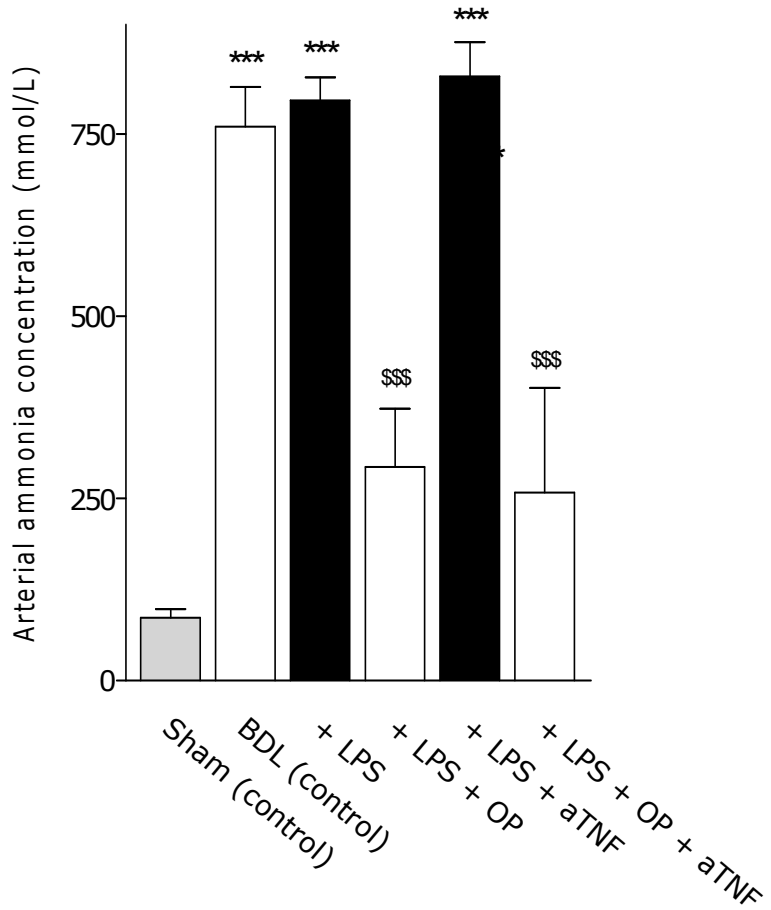
The Kaplan-Meier survival analyses were performed for the time to pre-coma/coma in the different treatment groups. Survival of BDL + saline compared to BDL + LPS rats was highly significant (Log-rank: $p<0.0001$). In BDL + LPS rats, OP treatment lead to significantly less progression to pre-coma/coma stages (Log-rank: $p<0.009$) which was not seen with Infliximab (Log-rank: $p=0.3$).

Figure 2. Frontal cortex brain water content



Shows that compared with sham-operated controls, there was a non-significant rise in frontal cortex water content in BDL rats, which was significantly augmented by LPS (**p<0.01). Administration of OP resulted in a significant reduction in brain water compared to LPS treated BDL rats ($^{\$\$}$p<0.01). Co-administration of OP + Infliximab, resulted in a significant reduction ($^{\$}$p<0.05) in brain water compared to LPS treated BDL rats. Infliximab had a non-significant effect on brain water. *Abbreviations: BDL, bile duct ligation; OP, L-ornithine, phenylacetate; LPS, lipopolysaccharide and aTNF, Infliximab.*

Figure 3. Changes in arterial ammonia



Shows that compared with sham-operated controls, there was a significant rise in arterial ammonia in BDL rats ($^{***}p<0.001$), with no additional effect with LPS. Following administration of OP (± Infliximab), there was a significant reduction in arterial ammonia concentration from baseline at 3 hours ($^{\$\$\$}p<0.001$, respectively) and no different to sham controls. *Abbreviations: BDL, bile duct ligation; OP, L-ornithine, phenylacetate; LPS, lipopolysaccharide and aTNF, Infliximab.*

Table 1

	Sham	BDL	BDL + LPS	BDL + LPS + OP	BDL + LPS + aTNF	BDL + LPS + OP + aTNF
Frontal brain water (%)	78.0 ± 0.1	78.7 ± 0.2	81.3 ± 0.6**	79.2 ± 0.4$^{\$\$}$	80.1 ± 0.4	78.5 ± 0.3$^{\$}$
Arterial ammonia (µmol/l)	86 ± 12	760 ± 55***	796 ± 32***	269 ± 108$^{\$\$\$}$	706 ± 169***	258 ± 143$^{\$\$\$}$
Bilirubin (mol/l)	16.0 ± 2.6	206 ± 3	212 ± 6	200 ± 9	195 ± 9	180 ± 18
Creatinine (mol/l)	20.5 ± 6.5	22 ± 1	24 ± 3	30 ± 2	26 ± 2	28 ± 0.0
Urea (mmol/l)	5.5 ± 0.4	5 ± 0.2	5 ± 0.6	6 ± 0.6	6 ± 0.3	5 ± 0.6
Albumin (g/l)	39 ± 1.8	35 ± 2	32 ± 1	35 ± 0.6	32 ± 1	30 ± 1
Total protein (g/l)	52.8 ± 2.8	46 ± 3	48 ± 2	45 ± 2	45 ± 2	54 ± 3
Sodium (mmol/l)	137 ± 4.0	139 ± 2	138 ± 3	140 ± 5	138 ± 1	141 ± 3
Potassium (mmol/l)	5.4 ± 1.0	4.9 ± 0.2	4.8 ± 0.6	5.1 ± 0.2	4.2 ± 0.2	5.9 ± 0.5
Chloride (mmol/l)	105 ± 4.4	104 ± 1	97 ± 2	108 ± 3	105 ± 2	106 ± 2
Osmolarity (mOsm/l)	285 ± 19.4	281 ± 20	279 ± 18	284 ± 20	280 ± 12	285 ± 28

Data are expressed as mean ± standard error of mean (SEM) Symbols represent;- $^{*}p<0.05$, $^{**}p<0.01$ and $^{***}p<0.001$ compared to sham-operated control rats; $^{\$}p<0.05$, $^{\$\$}p<0.01$ and $^{\$\$\$}p<0.001$ compared to respective saline treated control rat. Abbreviations: sham, sham-operated; BDL, bile duct ligation; LPS, lipopolysaccharide and aTNF, Infliximab.

Table 2

Cytokine levels (pmol/L)	Sham	BDL	BDL + LPS	BDL + LPS + OP	BDL + LPS + aTNF	BDL + LPS + OP + aTNF
Plasma TNF-α	90 ± 25	1859 ± 417*	4143 ± 528***/##	1919 ± 741$$	1371 ± 717$$$	179 ± 24$$$
Plasma IL-6	153 ± 52	1470 ± 422	4135 ± 560***/##	3730 ± 832	1462 ± 481$$	621 ± 267$$
Brain TNF-α	45 ± 13	179 ± 62	302 ± 68*	213 ± 38	188 ± 26	185 ± 38
Brain IL-6	26 ± 5	144 ± 99	226 ± 75	126 ± 83	156 ± 31	43 ± 19$

Data are expressed as mean ± standard error of mean (SEM) Symbols represent;- $^{*}p<0.05$, $^{**}p<0.01$ and $^{***}p<0.001$ compared to sham-operated control rats; $^{\$}p<0.05$, $^{\$\$}p<0.01$ and $^{\$\$\$}p<0.001$ compared to BDL + LPS rats and $^{\#}p<0.05$, $^{\#\#}p<0.01$ and $^{\#\#\#}p<0.001$ compared to BDL rats. Abbreviations: sham, sham-operated. BDL, bile duct-ligation. LPS, lipopolysaccharide. aTNF, Infliximab.

Research answers

1. Can a reduction in ammonia in cirrhotic rats prevent LPS induced worsening of brain oedema and progression to pre-coma and coma stages?

A reduction in arterial ammonia concentration with OP, prevent LPS induced worsening of HE and brain oedema, thereby priming the brain to the deleterious effect of LPS; though the mechanism of how ammonia primes the brain remains uncertain.

2. Does targeting hyperammonaemia and inflammation together provide therapeutic synergy in the treatment HE?

Although there was an observed advantage to combining ammonia-lowering and anti-inflammatory agents, there was no clear synergy, which may reflect the limitations of the specific inflammatory pathway used.

Chapter 5

Hepatic Encephalopathy:

Mechanistic study -

Role of Aquaporin 4 in liver failure

Published in '**Journal of Hepatology**' (2010)

Title:

Role of Aquaporin 4 in the Development of Brain Oedema in Liver Failure

Authors:

Wright G, Soper R, Brooks H.F, Stadlbauer V, Vairappan B, Davies NA, Andreola F, Hodges S.F, Moss R, Davies D.C, and Jalan R

Abstract

Liver failure is associated with progressive astrocyte swelling resulting in cytotoxic brain oedema, which is thought to underlie the pathogenesis of hepatic encephalopathy (HE). Although ammonia is considered to be central to the pathogenesis of HE, superimposed inflammation appears to play an important synergistic role in its pathogenesis. However, the mechanism underlying brain oedema in liver failure is unknown. The aim of this study was to determine whether aquaporin-4 (AQP4), an astrocyte bi-directional water channel, is involved in the brain oedema observed in liver failure. **Method:** (n=60) rats, received either sham-operation (sham), 5 days hyperammonaemia-inducing diet (HD), galactosamine (GALN) induced acute liver failure (ALF), 4 weeks' bile-duct ligation (BDL) induced cirrhosis, or caecal ligation and puncture (CLP), a 24 hour model of bacterial peritonitis. Rats in each of the groups (except CLP) were randomised to intraperitoneal (IP) injections of lipopolysaccharide (LPS; 1mg/kg) or saline, prior to termination 3 hours later. Brain water, AQP4 protein expression (western blot) and AQP4 localisation by Immunogold electron microscopy were investigated. **Results:** Significant hyperammonaemia was observed in BDL ($p<0.05$), GALN ($p<0.01$) and HD ($p<0.01$), compared to sham rats. LPS did not affect arterial ammonia and plasma biochemistry. Increased brain water was observed in GALN ($p<0.05$), CLP ($p<0.01$) and HD rats ($p<0.01$). Brain water was moderately increased in BDL rats, but this failed to reach significance ($p=0.09$). LPS treatment further increased oedema significantly in all groups ($p<0.05$, respectively). AQP4 expression was significantly increased in BDL ($p<0.05$), but not other rats, with maintained membrane polarisation in BDL rats. **Conclusion:** Our results suggest that AQP4 is not directly associated with the development of brain oedema in liver failure, hyperammonaemia or sepsis. In cirrhosis, there is increased AQP4 protein

expression with retained cellular polarisation possibly indicating a compensatory role to limit severe brain oedema.

Background

In patients with acute liver failure (ALF), about 30-50% progress to intracranial hypertension and cerebral edema.(267) On a background of hyperammonaemia and in the presence of retained blood brain barrier integrity, advanced HE is characterised by cytotoxic brain injury with astrocyte swelling; with severity of intracranial hypertension and mortality correlated to arterial ammonia levels, brain ammonia delivery and uptake.(116, 146) With cirrhosis, despite the presence of hyperammonaemia and a proinflammatory state,(331, 360) there is often only 'type 2' astrocytosis and occasional minimal brain swelling.(2, 150, 361, 362) However, following an episode of profound acute infection or aseptic inflammation, cirrhosis may decompensate to acute-on-chronic liver failure (ACLF),(7) with rapidly worsening cerebral oedema, similar to that observed with ALF. This may indicate that on a background of hyperammonaemia, type 2 astrocytosis predisposes the astrocyte to profound changes in cellular hydration following inflammation and/or osmotic stress. The mechanism of astrocyte oedema in the context of liver dysfunction and consequent hyperammonaemia has been hypothesised to be associated with an alteration in brain osmolytes, manifested by an accumulation of glutamine.(1, 177, 363, 364) The results of several lines of investigation support this hypothesis,(3, 107, 121, 149, 150, 307, 338, 361, 365) but the mechanism of worsening astrocyte oedema in the presence of an inflammatory insult in liver disease and hyperammonaemia is unclear. In a previous study, an inflammatory insult superimposed on a background of cirrhosis, was found to be associated with increased brain oedema and rapid progression to coma, but the blood-brain barrier and brain osmolytes remained apparently unaffected, suggesting that alternative mechanisms were likely to be important in the development of pathological brain oedema.(331)

In the brain, the bi-directional transmembrane water channel proteins - Aquaporin (AQP) 1, 4 and 9 assist in the regulation of cellular water homeostasis along osmotic gradients. AQP4 in particular is overwhelmingly expressed at astrocyte endfeet,(366) making it integral to blood-brain barrier integrity(367) and a potential factor in both vasogenic or cytotoxic oedema. The current literature supports a role for AQP4 in brain oedema associated with multiple forms of brain injury,(366, 368-371) although it remains unclear whether this is related to temporal and/or spatial changes in AQP4 expression,(372) especially in liver failure, about which data is limited to studies in isolated cultured astrocytes exposed to high concentrations of ammonia.(373-375) AQP4 has recently been implicated in the anti-oedema effect of hyperosmotic agents such as hypertonic saline in focal cerebral ischemia(376) but whether altered AQP4 is important in the pathogenesis of brain edema in liver failure is unknown. Furthermore, the role of AQP4 in cellular hydration may involve the activation (phosphorylation) of p38 Mitogen-activated protein kinase ($p38^{MAPK}$).(372)

Research hypothesis

The above observations have led to the hypothesis that AQP4 is an integral component in the development of pathogenic brain oedema in liver failure, especially following an inflammatory insult, via MAPK-dependent pathways and changes in cellular localisation.

Research questions

4. Are spatial and/or temporal changes in AQP4 involved in the development of pathogenic brain oedema in liver failure?

5. LPS augments cerebral oedema in HE; does this involve alteration in AQP4 expression as suggested in other disease states?

6. Does any effect of AQP4 expression on such brain oedema involve changes in MAPK expression?

Aim of Study

The aim of this study was to determine whether the development of brain oedema in models of liver failure is associated with altered AQP4 protein expression and cellular localisation, and whether this is influenced by the duration of liver injury, extent of hyperammonaemia, sepsis or aseptic inflammation. Levels of $p38^{MAPK}$ were also investigated in order to determine whether any alteration in AQP4 expression is associated with increased phosphorylation of $p38^{MAPK}$.

Methods

All animal experiments were conducted according to Home Office guidelines under the UK Animals and Scientific Procedures Act 1986. Male Sprague-Dawley rats (Charles-Rivers, UK), weighing 230–280g, were obtained from the Comparative Biology Unit at University College London. For the induction of the septic encephalopathy (caecal ligation & puncture; CLP), male Wister rats (Harlan Teklab, UK) weighing between 180–240g, were obtained from the Biological Research Facility at St Georges University of London. All rats were housed in the respective unit and given free access to standard rodent chow and water, serially weighed, with a light/dark cycle of 12 hours (the dark phase extended from 1900–0700 hours), at a temperature of 22–23°C and approximately 50% relative humidity.

Animal models

Bile-duct Ligation (BDL): (n=12) rats underwent BDL to induce biliary cirrhosis, or sham-operation (sham) (n=12) under anaesthesia - intravenous (IV) diazepam (1mg/kg), followed by a 150µl/kg of intramuscular Hypnorm® (Janssen Pharmaceutica, Belgium), as described previously.(306)

Galactosamine (GALN) induction of ALF rats: (n=12) rats received a single intraperitoneal (IP) injection of GALN (1mg/kg), 24 hours before sacrifice.

High protein (Ammoniagenic) diet (HD): (n=12) rats were administered an ammoniagenic high protein diet for 5 days prior to termination (as outlined above). The diet consisted of a liquid rodent feed (Bioserve, Frenchtown, NJ 08825, USA) and a tailor-made mixture mimicking the amino-acid composition of the haemoglobin molecule (4g/Kg/day Nutricia, Cuijk, The Netherlands) as described previously,(115, 307) and mixed with commercially available gelatin to prevent sedimentation. This regimen produces chronic hyperammonaemia.(331)

Caecal ligation & puncture (CLP): CLP is 24 hours rat model of bacterial peritonitis with sepsis, known to exhibit severe perimicrovessel oedema, swollen cortical astrocyte endfeet, due to both cytotoxic and vasogenic injury.(184) Six (n=6) rats underwent CLP under general anaesthesia to induce septic encephalopathy, and (n=6) underwent sham, as previously described.(184) General anaesthesia was induced by inhalation of a mixture of 0.6 l/min O_2 + 0.4 l/min N_2O and 4–5% Halothane (Zeneca Pharmaceuticals, UK). Anaesthetic agents are known to interact with a number of molecular sites, including glutamatergic and GABAergic receptors and uptake transporters for glutamate, GABA, and dopamine, among others; affecting the release of dopamine, acetylcholine, and L-glutamate and also mask drug-related effects on neurotransmitter release. CLP-shams were therefore used to rule-out a possible confounding effect of anaesthesia on these CLP rats compared to those used in the other study groups.

The rats were kept warm during surgery to prevent anaesthetic-induced hypothermia. The lower right quadrant of the abdomen was shaved and disinfected with 100% alcohol and the caecum mobilised through an approximately 2 cm long, right lower paramedian incision. For CLP rats, the caecum was filled by gently 'milking back' colon contents and then ligated below the ileo-ceacal valve without obstructing the continuity between the ileum and colon. The caecum was then subjected to a single 'through and through' perforation with a sterile 23-gauge needle and gently squeezed until its contents began to exude, to ensure patency of the perforation sites. For sham-CLP rats, the caecum was minimally handled without ligation and puncture. The bowel was then repositioned and the abdominal incision closed in layers with silk surgical sutures.

Study Design

In order to determine the effect of superimposed inflammation on the background of hyperammonaemia and/or liver dysfunction the treatment groups were given either saline or LPS. Four weeks after bile duct-ligation (or sham) rats were randomised, as were GALN and HD rats, to receive either an intraperitoneal (IP) injection of lipopolysaccharide (LPS; 1mg/Kg), or saline three hours before termination. A separate study was undertaken on the CLP rats approximately 24 hours after CLP, or sham-CLP. The final study groups were: 1) sham + saline (n=6), 2) sham + LPS (n=6), 3) GALN + saline (n=6), 4) GALN + LPS (n=6), 5) BDL + saline (n=6), 6) BDL + LPS (n=6), 7) HD + saline (n=6) 7) HD + LPS (n=6), 8) CLP (n=6) and 9) sham-CLP (n=6). Treatment groups 1) – 7) were given free access to food and water for 3 hours post-intervention in a temperature controlled environment and were then sacrificed by exsanguination under lethal anaesthesia IM Hypnorm (200μL/kg), 20 minutes after IP diazepam (1mg/kg). CLP and sham CLP rats (groups 8 and 9) were given free access to food and at water in a temperature controlled environment and approximately 24 h post-intervention were given a lethal dose of anaesthetic (Pentobarbital 35 mg/kg IP (Animalcare Ltd, York, U.K). Blood was withdrawn from the descending aorta of all lethally anaesthetised rats and immediately put into ice cold heparin/EDTA containing tubes, centrifuged at 3120 x g and 4^{o}C, and the plasma collected and stored at -80°C until assayed. Brain tissue was immediately harvested and collected and stored at -80°C until analysed.

Assessment of level of consciousness

The level of consciousness of all rats used in this study was rated prior to sacrifice using an established neurological scale.(308) It was rated as either normal, with loss of the scatter reflex (c.f. Grade 1 encephalopathy) and ataxia (c.f. Grade 2 encephalopathy), together representing pre-coma stages, or loss of the righting

reflex (c.f. Grade 3-4 encephalopathy) representing the coma stage. For CLP rats, behaviour and consciousness was measured as previously described.(184) In CLP rats, lethality is reported to be high, a humane endpoint was pre-set before the onset of lethality using behavioural and physiological parameters(377, 378) and measured as previously described.

Brain water

Immediately after death, the whole brain was rapidly removed and 50mm^2 samples of frontal cortex were taken. Cerebral cortex water content was determined using a previously described wet-to-dry weight ratio technique.(322, 334) Briefly, oven dried eppendorf's were weighed, then 50mm^2 wet frontal cortex samples placed into individual eppendorf's and reweighed; all samples weighed within 0.1 mg of each other. The dry weight of each sample of frontal cortex was determined after the brain-loaded eppendorf's were spun in a vacuumed eppendorf centrifuge at 10,000 x g and 40°C for 2 days. Tissue water content was then calculated as % H_2O = (1-dry wt/wet wt) x 100%.

Measurement of plasma biochemistry

Plasma samples (200µl) were analysed for: ammonia, alanine aminotransferase (ALT), albumin, total protein, bilirubin, urea and electrolytes using a Cobas integra 400 multianalyser with the appropriate diagnostic kits (Roche-diagnostics, Burgess Hill, West Sussex UK).

Western blot analysis

Snap frozen (-80°C) and stored 100µg cortical brain samples were homogenised and deproteinised (using a glass tube Teflon pestle homogeniser) in 300µl of ice-cold cell lysis buffer solution. After centrifugation at 12,000g for 10 minutes at 4°C, the supernatants were collected for processing. Following protein concentration quantification of equilibrated 50µl brain protein samples were analysed with protein

separation and transfer were performed using a NuPAGE® pre-cast gel system (Invitrogen Ltd, UK). Specific protein bands were detected using rat polyclonal IgG's for phosphorylated $p38^{MAPK}$ and total $p38^{MAPK}$ (Santa Cruz Biotechnology, Inc. USA) rabbit, anti-rat affinity purified polyclonal aquaporin-4 antibody (Chemicon International, U.K.) with a secondary goat polyclonal antibody to rabbit IgG, HRP conjugated (Hycult biotechnology, Netherlands). Alpha-tubulin (α-tubulin) was used as the housekeeping protein (Santa Cruz Biotechnology, Inc. USA), with a secondary goat polyclonal antibody to mouse IgG, HRP conjugated (Hycult biotechnology, Netherlands). All antibodies were used at a dilution of 1:1000. Protein bands were visualised using Amersham ECL™ advance western blotting detection reagents and Hyperfilm™ (GE Healthcare, UK). Densitometry measurements where made using Image-J software (freeware; rsbweb.nih.gov/ij).

To allow for accurate measurements of protein expression, it was necessary to take into account differences in total protein expression between sample tissues by corrected for the ubiquitous cellular cytoskeletal protein, α-tubulin.

Total $P38^{MAPK}$ and phosphorylated $P38^{MAPK}$' ratio: To reflect the relative activation of the $P38^{MAPK}$ pathway (indicated by phosphorylated $P38^{MAPK}$), the '$P38^{MAPK}$/$P38^{MAPK}$' ratio was calculated.

Aquaporin-4 Immunogold labelling

AQP4 localisation was assessed by Immunogold electron microscopy using a previously validated technique (performed by Dr G Wright and Dr Heather Brooks at the Institute of Hepatology, UCL and The Department of Anatomy, St Georges University, London). Sham and BDL rats under anaesthesia were perfused with 100 ml of phosphate buffer (0.1 M, pH 7.4) followed by 200 ml of 4% paraformaldehyde (in phosphate buffer), containing 0.2% glutaraldehyde and 4% sucrose. Brains were dissected out and 3 mm^3 pieces of frontal cortex were rinsed twice in phosphate

buffer for 15 minutes each time. Samples were then dehydrated in ascending alcohol (50%, 70%, 90%, 95%, 100%) for 30 minutes each before being left in a 2:1 ratio of ethanol:methacrolate overnight. The following morning samples were put into a 1:1 ratio of ethanol:methacrolate overnight. The following morning samples were put in a 1:1 ratio of ethanol:methacrolate for one hour before placing in pure methacrolate for 72 hours under UV light. Thin sections with a silver/gold interface colour (approximately 80-100 nm) were cut from the methacrolate embedded tissue blocks and collected on 200 mesh 3 mm nickel grids (Agar Scientific, U.K.). Sections were then rinsed in phosphate buffer (0.1M, pH 7.4) for 10 minutes before being placed in 10% goat serum (Invitrogen, Scotland, U.K.) for 30 minutes. Sections were then rinsed twice in phosphate buffer for 5 minutes each before being incubated overnight (4°C) in rabbit, anti-rat aquaporin-4 affinity purified polyclonal antibody (Chemicon International, U.K.), at a concentration of 1:250. Samples were again rinsed twice in phosphate buffer for 5 minutes each before being incubated for 1.5 hours (room temperature) in goat, anti-rabbit lgG (H&L) with 30 nm gold particles (British-biocell International, Cardiff, U.K.). Finally samples were rinsed twice with phosphate buffer (5 minutes each), rinsed once with distilled water (5 minutes) and stained with uranyl acetate. Grids were later viewed using a Hitachi 7100 transmission electron microscope at 75 kV. Digital images were captured with a Gatan column-mounted CCD camera at a resolution of 1024x1024 pixels and archived on a personal computer.

Statistics

Data are expressed as mean ± SEM. Treatment effects were investigated using Newman-Keuls multiple comparison test; $p<0.05$ was taken to be statistically significant. Software used included Microsoft Excel 2003 (Microsoft Corp., Redmond, WA) and GraphPad Prism 4.0 (GraphPad Software, Inc., San Diego, CA).

Results

There was no significant difference in the weight immediately prior to termination of GALN and CLP compared to sham-operated rats. There was no significant difference in the final weight of BDL and sham rats, and both groups continued to gain weight following surgery (mean BDL ± SEM = 320g ± 14 and mean sham ± SEM = 301g ± 19). The systemic haemodynamics in the BDL animals were well maintained as previously shown.(320, 331)

Neurobehavioral changes

All rats were alive 3 hours after injection of saline or LPS. Rats administered LPS showed a demonstrable effect with all displaying pilo-erection or hunched posture. The level of consciousness of BDL rats given LPS showed a marked, progressive deterioration, starting within 30 - 60 minutes and reaching pre-coma stages at the 3-hour killing time. Sham, GALN and HD rats administered saline and the sham and HD rats administered LPS remained fully alert. CLP lead to a reduction in activity indicative of lethargy (a diagnostic feature of systemic sepsis) without any evidence of grade 3 or 4 encephalopathy.(184)

Brain water measurements

There was a significant rise in frontal cortex water content in HD ($p<0.01$), GALN and CLP rats ($p<0.05$, respectively), compared to sham saline-treated controls. There was a numerical increase in cortical water content in BDL rats, but this just failed to reach statistical significance ($p=0.09$); Figure 1 and Table 1), consistent with previous findings with this model.(331) There was also a significant increase ($p<0.05$ for all comparisons) in the frontal cortex water content of LPS treated compared to the corresponding saline-treated (sham, GALN, HD and BDL) rats, Figure 1.

Arterial ammonia

When compared to sham controls there was a significant increase in plasma ammonia in BDL ($p<0.01$), GALN and HD rats ($p<0.01$) respectively; Figure 2 & Table 1). Induction of peritoneal sepsis by CLP had no significant impact on ammonia levels, nor did the administration of LPS in either sham, GALN, HD or BDL rats, compared to their saline treated counterparts. (Table 1)

Plasma biochemistry

Following BDL, there was a significant increase in bilirubin and a reduction in albumin consistent with cirrhosis, and an increase in ALT in the GALN animals ($p>0.001$), compared to sham rats, Table 2. Administration of LPS to sham, GALN, HD or BDL rats had no significant effect on plasma biochemistry except for a previously well-described increase in ALT and urea seen in LPS challenged BDL rats. Otherwise, renal function, sodium, potassium and plasma osmolarity were not statistically different between study groups.

Protein expression

AQP4 expression: There was a statistically significant increase in the expression of AQP4 following BDL ($p<0.05$; Figure 3) compared to sham rats, which was not observed following GALN-induced ALF or CLP. There was no significant effect of 5 days induced hyperammonaemia on AQP4 expression, when compared to sham controls. Administration of LPS to sham, GALN, BDL and HD rats also had no significant effect on AQP4 expression.

Phosphorylated-$P38^{MAPK}$/$P38^{MAPK}$ expression: There was a statistically significant increase in the expression of activated $p38^{MAPK}$ in BDL ($p<0.05$; Figure 4) compared to sham rats, which was not observed in GALN rats. In contrast, there was no significant increase in activated $p38^{MAPK}$ by induction of hyperammonaemia over 5 days in HD rats, with peritoneal sepsis in CLP rats, or any significant change in

activated $P38^{MAPK}$ levels with administration of LPS to sham, GALN, BDL or HD rats, compared to their saline treated counterparts.

Cellular localisation of AQP4 with Bile duct ligation and LPS administration

In view of the fact that increased AQP4 expression was observed in BDL rats, the ultra-structural localisation of this increased expression was investigated compared to sham rats. As previously reported,(331) the frontal cortex microvessels of BDL rats were partially collapsed (crescent shaped) with perivascular, compared with sham rats (Figure 5a, 5b). Immunogold labelling further demonstrated retained perivascular localisation of AQP4 in BDL rats with a demonstrable increase in levels. Although administration of LPS to BDL rats produced an increase in perivascular there did not appear to be any qualitative effect on AQP4 localisation (Figure 5c).

Discussion

The most important observations of this study were firstly to demonstrate that hyperammonaemia and/or inflammation, whether associated with liver dysfunction or not, causes an increase in brain water. Secondly, that the severity of brain swelling in these different models variably combining hyperammonaemia, liver dysfunction and inflammation are not associated with any increase in the expression of AQP4 in the brain. However, increased AQP4 protein expression was observed in BDL rats, but no further increase in AQP4 expression occurred in BDL rats treated with LPS (a model of ACLF), even though this resulted in a marked increase in brain water. These novel observations throw doubt on a causal role for AQP4 mediated brain oedema in not only liver failure, but also septic models.

The extent of brain water accumulation was profound in CLP, GALN, HD rats and LPS challenged BDL rats, with no correlation between AQP4 levels and extent of cerebral oedema. The only intervention that showed an increase in AQP4 protein expression was BDL, which manifests low grade brain oedema(150, 361) (with LPS not having any additional effect).

Immunogold labelling of thin electron microscopic sections,(379, 380) involves conjugation of gold particles to either primary of secondary antibodies for specific (especially surface) antigen detection. This is a well-established method for the cellular localization of AQP4,(381) with studies confirming that over 95% of brain AQP4 is concentrated in the membrane of astrocyte endfeet.(382) Importantly, Immunogold labelling confirmed the increased AQP4 expression found on western blotting, but also demonstrated that BDL has no effect on the ultra-structural localisation of AQP4. The literature on AQP4 expression following brain insult is confusing and is principally derived from models of focal brain ischaemic (FBI). Some studies have shown AQP4 to be down-regulated within 24 hours of FBI, when

brain water accumulation has occurred,(369, 383) although in another study(384) AQP4 mRNA was reported to be elevated, during resolution of the cerebral oedema (but only by day 3). A temporal and spatial alteration in AQP4 expression and any relationship to the site of water accumulation has also been shown to be highly variable in longitudinal studies.(385) In a key longitudinal study of AQP4 expression following FBI over days, AQP4 expression was shown to be increased at 1 and 48 hours post ischaemia, correlating with the peaks of brain water accumulation.(385) In a similar model, AQP4 down-regulation was reported to have occurred at the centre of ischaemic injury as early as 90 min post insult, peaking at 24 hours during the reperfusion phase, with partial recovery by 72 hours; though interestingly AQP4 expression was up-regulated at the border of injury.(386) Water intoxication (IP water and 8-deaminoarginine vasopressin), significantly increases AQP4 levels in wild-type mice after just 15 min, but this increase does not occur in similarly challenged AQP4 knockout mice that appear to be protected from brain oedema.(368, 387) Furthermore, the vasogenic brain oedema that occurs in wild-type mice following tumour cell implantation is not apparent in AQP4 knockout mice. Yet it seems improbable that demonstrable AQP4 protein turnover occurs within a matter of minutes or hours to induce pathogenic oedema, unless there was a sizable alteration to an existing cellular pool or functional modulation. Unless there is an increase in AQP4 levels and transmembrane polarisation before brain water accumulation, it is difficult to conclude a causal role for AQP4. A recent study showed that increasing AQP4 protein expression by 2-fold in astrocytes of AQP4-overexpressing transgenic (Glial fibrillary acid protein (GFAP)-AQP4 positive) mice, had no affect on survival, behaviour, or anatomy and did not induce brain oedema and intracranial hypertension, unless accompanied by a water-intoxification.(388) These findings imply that AQP4 upregulation may simply provide the environment

for further insult (e.g. osmotic). This is particularly pertinent to any possible association with liver disease with the importance of shifts in intracellular osmolytes in astrocytes as part of the 'ammonia-glutamine-brain swelling hypothesis'. The only prior study of AQP4 expression in relation to liver failure has been in astrocyte cell cultures incubated with very high concentrations of ammonia (5 mmol, ammonium chloride) where increased AQP4 expression was observed 2 hours before astrocyte swelling (10 hours post exposure) and persisted for 48 hours.(374) This swelling is thought to be due to oxidative stress induced changes in MAPK-pathways.(375) However, incubating astrocytes with ammonium chloride does not fully model the effects of liver failure on the brain, because it involves an unphysiological osmotic stress and therefore, does not allow extrapolation of a causal role for AQP4 in the brain oedema of liver failure.

It is likely that the increased AQP4 expression in BDL rats in the current study is a compensatory mechanism to minimise pathogenic oedema as hypothesised in earlier reports of the role of AQP4 in cerebral oedema (e.g. vasogenic cerebral oedema associated with brain tumour and contusion injury(389) and bacterial abscess (390)).

The results of the current study also provide further evidence to indicate that brain swelling is not a surrogate for HE. Indeed, marked increases in brain water in several groups of animals was not associated with changes in mental state and our results also confirmed that superimposed inflammatory stress induced by LPS on the background of liver disease (both BDL and GALN) was associated with progression to pre-coma stages. The results of this study argue against a role for AQP4 as the key causal factor in pathogenic brain swelling. However, much of the evidence in support of this view is based on studies utilising AQP4 knockout mice (e.g. deletion of the anchoring protein α-Syn$^{(-/-)}$ which decreases the AQP4

perivascular pool by >90%) and AQP4-overexpressing transgenic mice.(366, 369-371, 376, 385, 387, 388, 390-392) However, data derived from such physiologically unrepresentative models should be treated with caution, because they may also exhibit possible confounding effects (e.g. changes in blood brain barrier integrity(367, 376)). Indeed, α-Syn$^{-/-}$ mice have less brain water than wild type mice (α-Syn$^{+/+}$)(376) and with such differing baseline control animals; it is difficult to assess changes in brain water following interventions (e.g. administration of hypertonic saline). Furthermore, the fact that that AQP4 over-expression only causes oedema with hyperosmolar insult(388) appears counterintuitive if AQP4 plays a pivotal causal role in brain oedema. Further doubt is cast upon a role for increased AQP4 expression causing cerebral oedema, by the results of a pathology study of AQP4$^{-/-}$ knockout mice, which demonstrate that the absence of perivascular AQP4 expression is associated with open inter-endothelial cell tight junctions and swelling of perivascular astrocyte endfeet, reducing blood-brain barrier integrity.(367)

It is important to point out that in the current study there was no confounding effect of plasma electrolytes between the treatment groups. This observation is important, because it could be argued that shifts in plasma K^+ levels may be integral to AQP4-dependent cellular hydration, via its close association with the K^+ inward rectifying (Kir-4.1) transporter, although the impact of this transporter has recently been questioned.(393) Another finding of the current study was that only bile duct-ligation lead to an increase in activated p38^{MAPK}. Given that increased AQP4 expression was also only caused by BDL, there appears to be an association between p38^{MAPK} activation and AQP4 expression. A similar association has been reported to occur in cultured astrocytes under conditions of hyperosmolar

stress(372) and suggests a role for MAP Kinase intracellular signaling (with resultant downstream nuclear factor kappa beta(394)) in AQP4 regulation.

In conclusion, AQP4 does not appear to play a significant role in the development of brain oedema associated with hyperammonaemia or inflammation, either with or without acute liver dysfunction. However, the brain oedema in cirrhosis is associated with upregulation of AQP4 with contemporaneous $p38^{MAPK}$ activation and retained cellular polarisation, possibly as a compensatory response to inhibit oedema formation.

Table 1

	Sham	Sham + LPS	GALN	GALN + LPS	BDL	BDL + LPS	HD	HD + LPS	sham CLP	CLP
Frontal brain water (%)	78.3 ± 0.3	79.8 ± 0.3$	79.9 ± 0.4*	80.2 ± 0.2*$	79.5 ± 0.3	80.8 ± 0.2$	81.1 ± 0.1**	81.8 ± 0.2**	79.6 ± 0.2	81.2 ± 0.2*
Arterial ammonia (µmol/l)	63 ± 7	67 ± 6	201 ± 20**	182 ± 21*	188 ± 16*	172 ± 36*	209 ± 17**	220 ± 32**	67 ± 4	71 ± 6

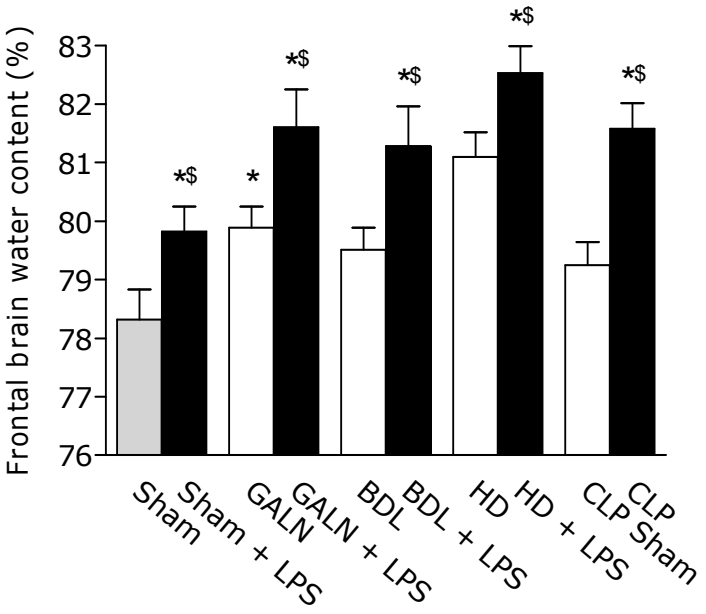
Data are expressed as mean ± standard error of mean (SEM). * = $p<0.05$, ** = $p<0.01$ and *** = $p<0.001$ compared to sham-operated control rats; $^{\$}$ = $p<0.05$, $^{\$\$}$ = $p<0.001$ and $^{\$\$\$}$ = $p<0.001$ compared to their respective saline-treated controls. Abbreviations: sham, sham-operated; GALN, galactosamine; BDL, bile duct ligation; HD, high-ammonia diet; LPS, lipopolysaccharide and CLP, caecal ligation & puncture.

Table 2

	Sham	Sham + LPS	GALN	GALN + LPS	BDL	BDL + LPS	HD	HD + LPS	CLP sham	CLP
Plasma ALT (U/l)	70 ± 6	90 ± 12	1505 ± 500**	1828 ± 294**	98 ± 15	168 ± 54	31 ± 2	37 ± 4	60 ± 12	132 ± 40
Plasma bilirubin (mol/l)	10 ± 3	24 ± 6	19 ± 9	17 ± 9	180 ± 18**	181 ± 14**	28 ± 3	32 ± 3	11 ± 3	12 ± 4
Plasma creatinine (mol/l)	22 ± 1	24 ± 3	39 ± 2	49 ± 2	18 ± 0.0	27 ± 5	39 ± 2	32 ± 2	18 ± 0	18 ± 0
Plasma urea (mmol/l)	5 ± 0.2	5 ± 0.6	6 ± 0.6	6 ± 0.3	5 ± 0.6	9 ± 0.4	7 ± 0.6	6 ± 0.9	6 ± 1	8.9 ± 1.2
Plasma albumin (g/l)	35 ± 2	38 ± 1	35 ± 0.6	38 ± 1	26 ± 1	24 ± 2	39 ± 0.7	40 ± 0.7	39 ± 0.7	40 ± 0.3
Plasma total protein (g/l)	46 ± 3	48 ± 2	45 ± 2	45 ± 2	54 ± 3	48 ± 3	51 ± 0.9	51 ± 1	43 ± 5	40 ± 3
Plasma sodium (mmol/l)	139 ± 2	138 ± 3	140 ± 5	138 ± 1	141 ± 3	141 ± 3	136 ± 5	139 ± 5	139 ± 5	137 ± 4
Plasma potassium (mmol/l)	4.9 ± 0.2	4.8 ± 0.6	5.1 ± 0.2	4.2 ± 0.2	5.9 ± 0.5	6.0 ± 0.3	5.2 ± 0.4	5.1 ± 0.1	5.0 ± 0.2	5.9 ± 0.5
Plasma chloride (mmol/l)	104 ± 1	97 ± 2	108 ± 3	105 ± 2	106 ± 2	105 ± 3	97 ± 0.9	99 ± 0.9	102 ± 1	102 ± 1
Plasma osmolarity (mOsm/l)	281 ± 20	279 ± 18	284 ± 20	280 ± 12	285 ± 28	288 ± 23	278 ± 23	284 ± 36	283 ± 20	280 ± 19

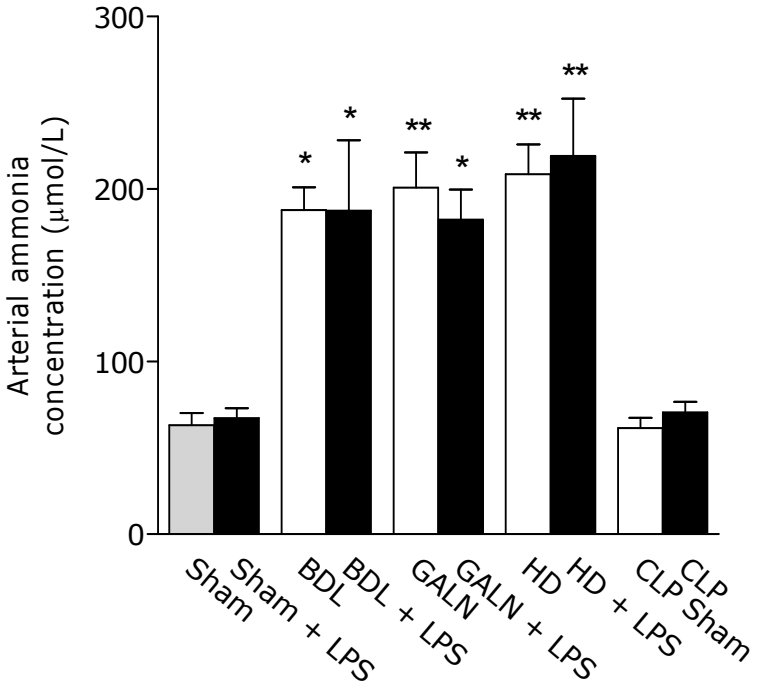
Data are expressed as mean ± standard error of mean (SEM). * = $p<0.05$, ** = $p<0.01$ and *** = $p<0.001$ compared to sham-operated control rats; $^{\$}$ = $p<0.05$, $^{\$\$}$ = $p<0.01$ and $^{\$\$\$}$ = $p<0.001$ compared to their respective saline-treated controls. Abbreviations: sham, sham-operated; GALN, galactosamine; BDL, bile duct ligation; HD, high-ammonia diet; LPS, lipopolysaccharide and CLP, caecal ligation & puncture.

Figure 1: Frontal cortex brain water content



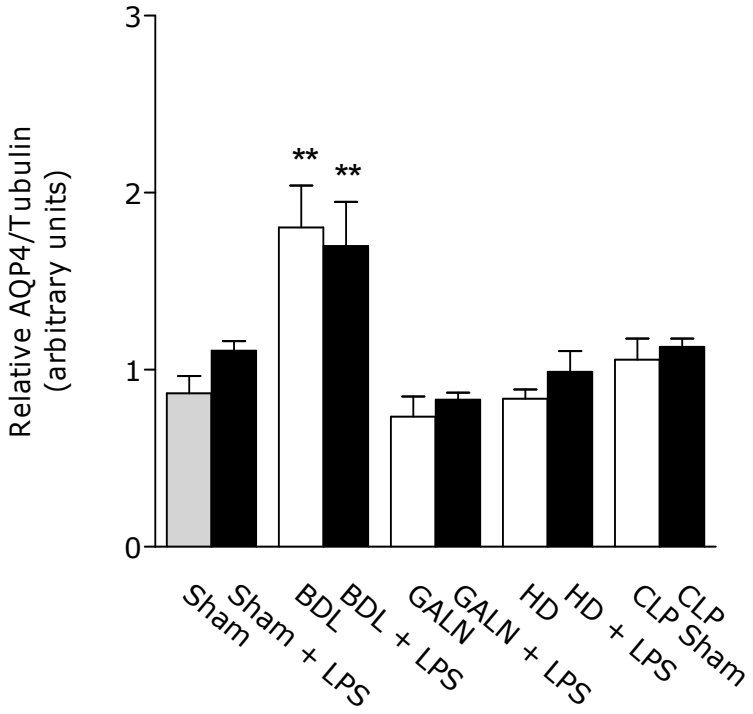
There was significant brain swelling in galactosamine (GALN), cecal ligation and puncture (CLP) and high-ammonia diet (HD) rats, but bile duct-ligation (BDL) rats administered saline, were not significantly different from sham-operated (sham) rats. Lipopolysaccharide (LPS) significantly augmented brain swelling in all treatment groups ($p<0.05$, for all comparisons). * = $p<0.05$, compared to sham rats; $ = $p<0.05$, compared to respective saline-treated controls

Figure 2: Plasma ammonia levels



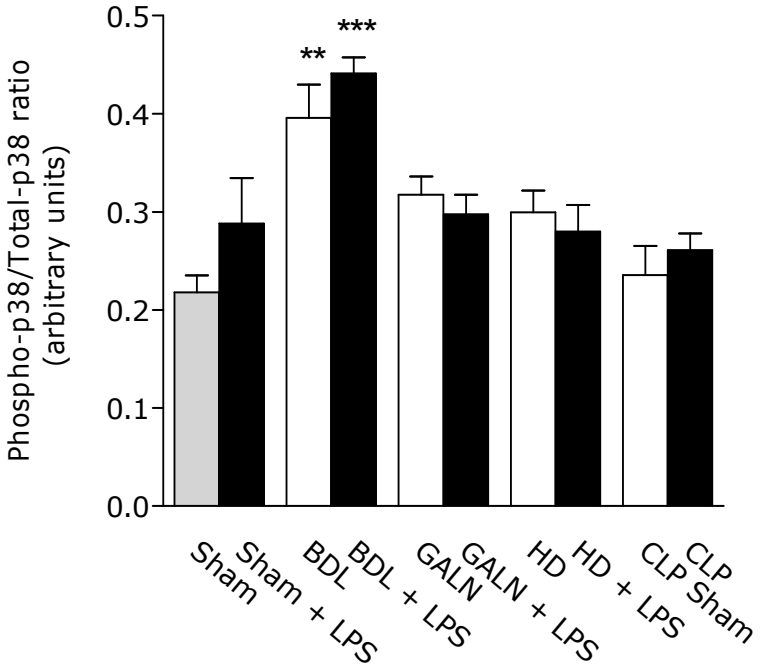
There was a significant rise in plasma ammonia in bile duct ligation (BDL), galactosamine (GALN) and high-ammonia diet (HD) rats. Administration of lipopolysaccharide (LPS) or induction of sepsis by cecal ligation and puncture (CLP) had no demonstrable additional effect on ammonia levels. * = $p<0.05$ and ** = $p<0.01$ compared to sham-operated (sham) control rats.

Figure 3: AQP4 levels in the frontal cortex



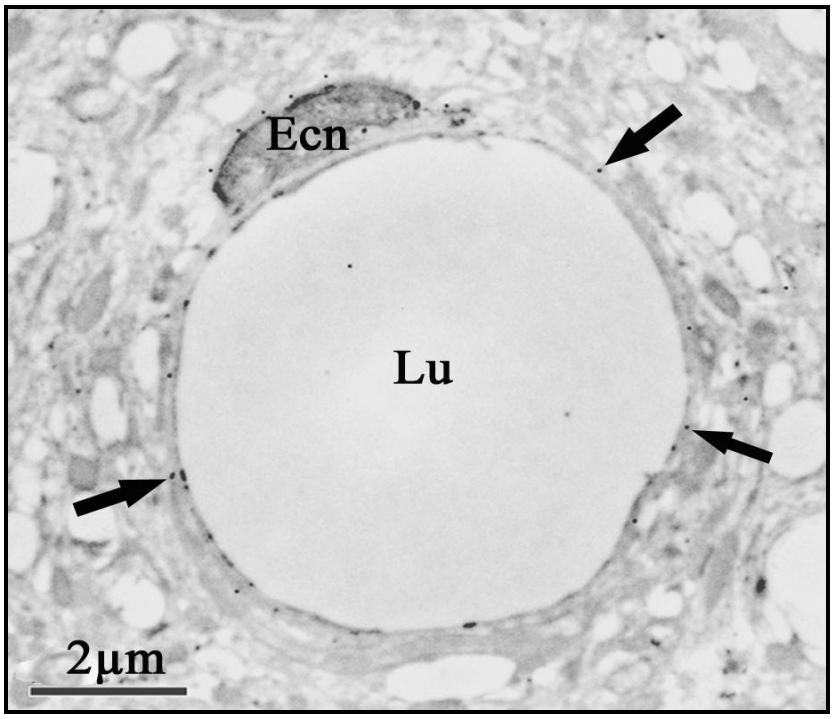
There was a significant increase in AQP4 protein expression in bile duct ligation (BDL) rats that was not observed with galactosamine (GALN), high ammonia diet (HD) or cecal ligation and puncture (CLP) rats. Administration of lipopolysaccharide (LPS) had no effect on AQP4 expression. * = $p<0.05$, ** = $p<0.01$ and *** = $p<0.001$ compared to sham-operated (sham) control rats; $^{\$}$ = $p<0.05$, $^{\$\$}$ = $p<0.001$ and $^{\$\$\$}$ = $p<0.001$ compared to respective saline-treated controls.

Figure 4: Changes in p38^{MAPK} activation



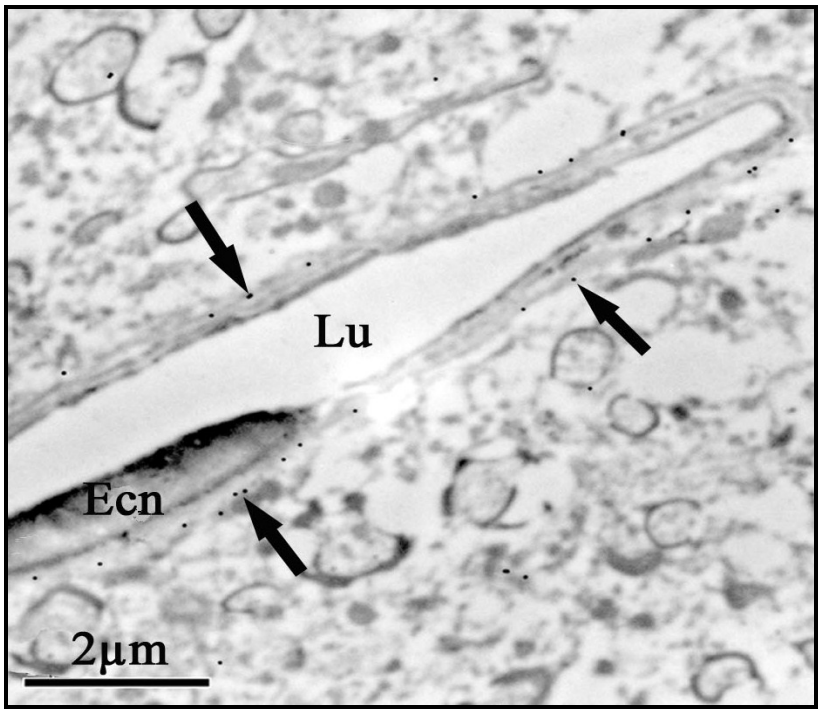
There was a significant increase in p38^{MAPK} activation in bile duct ligation (BDL) rats that was not observed with galactosamine (GALN), high-ammonia diet (HD) or cecal ligation and puncture CLP. Administration of lipopolysaccharide (LPS) had no effect on p38^{MAPK} activation. * = $^{*}p<0.01$ and *** = $p<0.001$ compared to sham-operated (sham) control rats.

Figure 5a: Representative Immunogold labelled transmission electron micrograph of a brain microvessel from the frontal cortex of a sham-operated rat



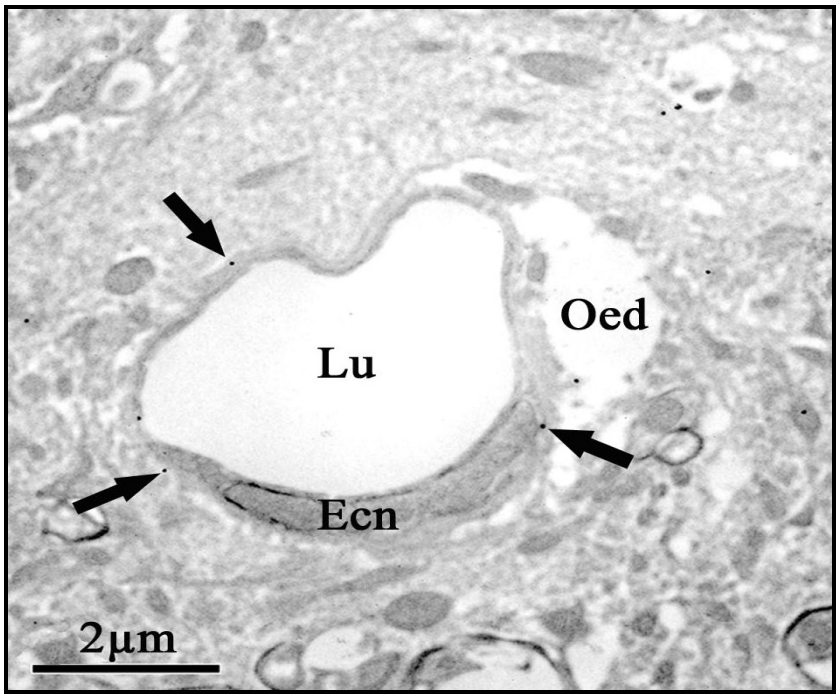
This shows an intact and well-perfused microvessel with no surrounding astrocyte, perivascular changes. AQP4 immunogold (Au) labelling, detected as black dots (see arrows), can be seen to decorate astrocyte endfoot membranes in contact with the abluminal surface of the microvessel. *Abbreviations: Ecn, Endothelial cell nucleus, Au, gold particle, Oed, oedema.*

Figure 5b: Representative Immunogold labelled transmission electron micrograph of a brain microvessel from the frontal cortex of a bile duct ligation rat



This shows a collapsed (crescent-shaped) microvessel with an intact endothelial barrier with minimal perivascular oedema. AQP4 immunogold (Au) labelling, detected as black dots (see arrows), shows retained perivascular polarization of AQP4 in astrocyte endfeet processes, in greater abundance than in sham-operated rats. *Abbreviations: Ecn, Endothelial cell nucleus, Au, gold particle, Oed, oedema.*

Figure 5c: Representative Immunogold labelled transmission electron micrograph of a brain microvessel from the frontal cortex of a LPS challenged BDL rat



This shows a partially collapsed microvessel with profound perivascular oedema (Oed). AQP4 immunogold (Au) labelling, detected as black dots (see arrows), shows retained perivascular polarization of AQP4, similar to that in BDL saline treated controls. *Abbreviations: Ecn, Endothelial cell nucleus, Au, gold particle, Oed, oedema.*

Research answers

1. Are spatial and/or temporal changes in AQP4 involved in the development of pathogenic brain oedema in liver failure?

AQP4 is not involved in the development in pathogenic brain oedema in liver failure, sepsis and hyperammonaemic rat models.

2. LPS augments cerebral oedema in HE; does this involve alteration in AQP4 expression as suggested in other disease states?

AQP4 is not involved in the development in LPS triggered pathogenic brain oedema.

3. Does any effect of AQP4 expression on such brain oedema involve changes in MAPK expression?

MAPK expression has a contemporaneous association with AQP4 expression.

Chapter 6

Hepatic encephalopathy: The role of Inflammation, Ammonia and Aquaporin Expression in the Pathogenesis of Cerebral Oedema

PhD Thesis Summary

Introduction

This thesis was undertaken to expand our current understanding of interorgan ammonia and amino acid metabolism in the pathogenesis of hepatic encephalopathy (HE); discussed in chapter one. A review of HE literature highlighted a number of key unanswered questions, chiefly focused on the mechanisms underlying the possible synergy between ammonia and inflammation. Utilising animal and laboratory experiments, this thesis comprehensively explored a number of concise hypotheses (chapter 2-5). The findings from these key studies are summarised as follows:

STUDY 1 (Chapter 2)

Current evidence indicates synergy between hyperammonaemia and inflammation in the development of Hepatic encephalopathy (HE). However, it is not clear whether it is the background cirrhotic state or the associated hyperammonaemia that predisposes to the effects of superimposed inflammation.

Question 1*:* In bile duct-ligated (BDL) rats, does lipopolysaccharide (LPS) induced systemic inflammation worsen the brain oedema of cirrhosis? If so, is this associated with blood-brain barrier disruption? Altered brain ammonia metabolism? or modulation of inflammatory brain responses?

Answer 1*:* LPS derived systemic inflammation triggered brain oedema in sham-operated control rats worsened the marked oedema observed in ammonia fed naïve rats and the pre-existing low-grade oedema (consistent with minimal HE) observed in cirrhotic rats. However, despite the extent of oedema induced by ammoniagenic feed (and in rats given LPS), only cirrhotic rats challenged with LPS progressed to

pre-coma/coma. Furthermore these changes occurred in the context of retained barrier integrity, indicative of cytotoxic oedema. However, plasma and brain ammonia levels were high in both ammonia fed naïve and cirrhotic rats and both observed worsening brain oedema with the acute inflammatory insult of high dose LPS, though LPS did not independently alter plasma or brain ammonia levels. An explanation for the difference in coma levels between these 2 groups, which both display the phenotype of background high circulating ammonia and acute inflammation, may be related to the chronicity ('time to development') of significant hyperammonaemia - chronic (BDL) or acute (ammonia fed) rats. The slower rise in ammonia with the secondary cirrhosis of BDL, may allow for a relative protection against intracellular osmolyte shifts consistent with the 'ammonia-glutamine-brain water' hypotheses. This hypothesis describes the brain edema of hyperammonaemia in terms of alterations in brain osmolytes, characterised by increases in the brain glutamine/myo-inositol ratio. In ammonia fed naïve rats (that had markedly more oedema than cirrhotic BDL rats), there was a more significant increase in the brain glutamine/myo-inositol ratio than that observed with BDL rats. The acute systemic proinflammatory (cytokine and nitric oxide) stimulus induced by LPS, augmented a background proinflammatory cytokine response (e.g. Tumour necrosis factor-alpha (TNF-α) and Interleukin-6 (IL-6)) in cirrhotic BDL rat brain, not evident in other groups. Interestingly, only comatose LPS challenged BDL rats showed high levels of brain nitrotyrosine, possibly suggesting that hyperammonaemia and inflammation on a background of cirrhosis provide an environment for nitrosation of brain proteins. The LPS challenged BDL rat is therefore the first small animal model which mimics the clinical presentation of 'acute-on-chronic liver failure' (ACLF) and also provides support for the concept of

synergy between ammonia and inflammation in the development of brain oedema and progression to coma in cirrhotic rats.

In conclusion, injection of LPS into cirrhotic BDL rats induces pre-coma/coma, and exacerbates cytotoxic oedema due to the synergistic effect of hyperammonaemia and induced inflammatory responses. Although the exact mechanism of how hyperammonaemia and LPS facilitate cytotoxic oedema and pre-coma/coma in cirrhosis is not clear, this data supports an important role for the nitrosation of brain proteins.

STUDY 2 (Chapter 3)

In patients with cirrhosis, new therapies targeting hyperammonaemia and HE are necessary. A novel new drug, L-ornithine Phenylacetate (OP), which combines two known ammonia-lowering agents, is hypothesised to improve ammonia reduction, but the exact mechanisms of its beneficial action have yet to be fully explored.

Hypothesis

In hyperammonaemic BDL rats, the combination of L-ornithine and phenylacetate as OP:

1) Traps ammonia as glutamine within muscle by provision of L-ornithine to muscle glutamine synthetase
2) L-ornithine derived glutamine is excreted by conjugation with phenylbutyrate (converted to phenylacetate) to phenylacetylglutamine

This means that ammonia will not be available for later return to the circulation, resulting in net removal and reduction in ammonia concentration.

Question 2: Does L-ornithine and phenylacetate (when combine as OP), act synergistically to provide an improved and more sustained reduction in ammonia? Is this achieved by L-ornithine acting as a substrate for glutamine synthesis thereby detoxifying ammonia? and/or the phenylacetate excreting the ornithine derived glutamine as phenylacetylglutamine in the urine?

Answer 2: BDL produces high arterial ammonia, brain oedema and lower brain *myo*-inositol consistent with secondary cirrhosis. When compared to lone therapy with either L-ornithine or phenylacetate, the administration of OP was associated with a more significant and sustained reduction in arterial ammonia. The reduction in ammonia with administration of OP occurs within hours and is associated with an initial increase in circulating ornithine at 1 hour, and corresponding rise in circulating glutamine at 3 hours as ornithine falls. These findings are in accordance with the first part of the hypothesis, with such changes in glutamine levels consistent with previous observations of increased skeletal muscle glutamine synthetase activity. The reduction in ammonia with OP was associated with a significant reduction in the brain glutamine/myo-inositol ratio and prevention of brain oedema, principally by maintaining higher brain myo-inositol levels. This implies an increase in the brain buffering capacity to the effects of other precipitants such as further hyperammonaemia or hyponatraemia that are known to increase brain swelling. The beneficial effects of OP were sustained during a 10 day period of administration with respect to limiting hyperammonemia and also brain oedema, without any evidence of a rebound increase in ammonia over this period. In accordance with the proposed hypothesis, glutamine generated by the administration of L-ornithine was conjugated and excreted as phenylacetylglutamine by co-administration of phenylbutyrate.

In conclusion: The results of this study provide proof of concept that L-ornithine and phenylbutyrate act synergistically to produce sustained improvement in arterial ammonia, its brain metabolism and brain water. As L-ornithine and phenylbutyrate are already in use in man, it is likely that these findings can be translated relatively quickly into appropriate clinical studies to determine exact dosing, pharmacokinetics, safety and efficacy.

STUDY 3 (Chapter 4)

In liver failure, inflammation synergistically modulates the cerebral effects of ammonia, with interventions targeting either hyperammonemia or inflammation limiting progression of HE.

Question 3: Can a reduction in ammonia in cirrhotic rats prevent LPS induced worsening of brain oedema and progression to pre-coma and coma stages? Does targeting hyperammonaemia and inflammation together provide therapeutic synergy in the treatment HE?

Answer 3: The results of this study show for the first time that reduction in arterial ammonia concentration using OP in BDL rats is associated with a reduction in LPS induced progression to pre-coma/coma which is associated with a marked attenuation in brain swelling. These data provide a strong argument in support of the hypothesis that, on the background of chronic liver disease (BDL), ammonia may prime the brain to the deleterious effect of LPS. As predicted, administration of Infliximab was associated with marked attenuation of the pro-inflammatory drive manifested by a reduction in arterial TNF-α and IL-6 concentrations without an effect on ammonia levels, but this was not associated with any significant reduction

in brain oedema. Co-administration of OP and Infliximab had similar effects to that observed in the OP treated group, thus failing to demonstrate any significant therapeutic synergy between OP and Infliximab. These observations provide proof for the concept that ammonia may in some way 'prime' the brain to the effects of LPS. These findings, along with recent data from our group showing differential TLR expression (and thus innate immunity) in the brain of BDL rats, suggesting that ammonia might prime the brain to the effects of microbial challenge via effects on TLR expression.

In conclusion*:* the findings of this study substantiate the predominant role of ammonia in priming the brain to the deleterious effect of LPS and suggest that a reduction in arterial ammonia concentration with OP may prevent LPS induced worsening of HE and brain edema. The mechanism of how ammonia primes the brain remains uncertain but our results supports the role for TLR.

STUDY 4 (Chapter 5)

Progressive astrocyte swelling as the underlying pathogenic process of cytotoxic brain oedema is considered critical to advanced HE. Though ammonia is thought central to its pathogenesis, superimposed inflammation plays an important synergistic role, often provoking brain oedema. Yet the mechanism of brain oedema in liver failure is unknown. Current literature, suggests a possible role for aquaporin-4 (AQP4), a bi-directional water channel predominant in astrocytes, in the development of brain oedema in a number of neuropathic disorders.

Question 4*:* Is AQP4 causal in the brain oedema associated with various models of liver failure?

The study incorporated the following models:

- BDL (cirrhotic) rats
- BDL + LPS challenged (ACLF) rats
- Galactosamine (Acute liver failure (ALF)) rats
- Ammonia fed naïve (hyperammonaemic) rats
- Caecal ligation and puncture (CLP) rats; a 24 hour model of sepsis

Answer 4*:* This study firstly confirms that hyperammonaemia and/or inflammation, whether associated with liver dysfunction or not, causes an increase in brain oedema. Secondly, that the severity of brain swelling in these different models, which variably combine hyperammonaemia, liver dysfunction and inflammation, are not associated with any increase in the expression of brain AQP4, except for in cirrhotic rats. In cirrhotic rats (which manifest low grade brain oedema), increased AQP4 protein expression was observed, with a clear contemporaneous association to $p38^{MAPK}$ activation. With progression to cirrhosis, there was an apparent temporal, but not spatial change in the distribution of AQP4 along perivascular astrocytes (using Immunogold labelling). The addition of LPS to cirrhotic rats (a model of ACLF) had no effect on AQP4 protein expression, seriously questioning the role of AQP4 in the pathogenesis of brain oedema in these models. The results of this study also provide further evidence to indicate that brain swelling is not a surrogate for HE. Indeed, marked increases in brain water in several groups of animals was not associated with changes in mental state and our results also confirmed that superimposed inflammatory stress induced by LPS on the background of liver disease (both BDL and GALN) was associated with progression to pre-coma stages.

In conclusion, AQP4 does not appear to play a significant role in the development of brain edema associated with hyperammonemia or inflammation, either with or without acute liver dysfunction. However, the brain edema in cirrhosis is associated with upregulation of AQP4 with contemporaneous $p38^{MAPK}$ activation and retained cellular polarisation, possibly as a compensatory response to inhibit edema formation.

Conclusion

The body of work within this thesis demonstrates the critical nature of interorgan ammonia and amino acid metabolism and the relationship that exists between ammonia and inflammation in the pathogenesis of HE. This research has also shown that systemic inflammatory mediators modulate cerebral inflammatory processes, which are likely primed by the effects of ammonia on the brain. Also this thesis helps dispel any belief of a causal role for AQP4 expression in the development of brain oedema in HE (and likely other neuropathic conditions). Understanding the importance of interorgan ammonia and amino acid metabolism has lead to the development of a novel drug - L-ornithine, phenylacetate (OP), which may provide us with the first clinically useful intervention for this life-threatening condition. As L-ornithine and phenylbutyrate are already in use in man, it is likely that these findings can be translated relatively quickly into appropriate clinical studies to determine exact dosing, pharmacokinetics, safety and efficacy. Furthermore, to test the hypothesis of synergy this thesis has for the first time studied the application of combining therapies targeting both ammonia and inflammation. This thesis concludes that while hyperammonaemia remains the chief culprit and main therapeutic target in the treatment of HE, as it also appears to prime cells to the effect of inflammation, a better understanding of its potentially synergistic interaction with inflammatory processes may yet prove a worthwhile therapeutic goal in the foreseeable future.

Acknowledgements

I am sincerely grateful for the opportunity afforded me to undertake this PhD thesis. This period of research has encouraged focus and discipline, while providing many necessary skills applicable to my wider career. This body of work has allowed me to travel extensively to present at international meetings, publish a number of high impact, peer-reviewed science papers and cultivate important and ongoing collaborations.

The principle person I must unreservedly thank is my supervisor and mentor Professor Rajiv Jalan. I have always been cognisant that Professor Jalan was offering me a great opportunity to undertake a period of significant research at one of the country's most distinguished higher seats of learning. Having provided the '*paradigm*' for my research ideals, I am appreciative of our insightful inflections, born out of our mutual passions towards our work. I have been fortunate to be a part of the Liver Failure Group, at the Institute of Hepatology, University College London (UCL), with its clinical attachment to University College Hospital. This has allowed me to complete large clinical studies, while acquiring a wide range of laboratory and animal experimentation skills. There has been a constant stream of highly educated colleagues at the institute, many of whom are now friends. Of particular mention should be Dr Raj Mookerjee (Hepatologist, UCL), with his articulate prose and unmistakable research vocabulary. I have also worked alongside and continue to collaborate with Dr Debbie Shawcross (Hepatologist, King's College London) and Dr Vanessa Stadlbauer (Medical University of Graz, Austria). Both taught me flow cytometry and helped me to develop cell-culture studies. They have been at the foundation of any success I may attribute to my time at the Institute. I would also like to single out Dr Stadlbauer, Stephen Hodges

and Nathan Davies for their continued technical support. Stephen Hodges in particular, actively encouraged my research ideas and made every attempt to provide opportunities for me to develop each and every one. Whether offering insightful dialogue, or inane diatribe, as a group of individuals they have been a pleasure to work alongside.

I would like to say a special thank you to my second supervisor Dr Ceri Davies (now of Imperial College, London) who along with his associates at the Department of Anatomy, St Georges University of London, taught me key immunohistochemical and electron microscopic techniques pivotal to this thesis. Also, Dr Andrey Abramov in the Department of Physiology (UCL), for teaching me astroglial culture techniques. Having spent a period of time in the Department of Academic Surgery at the University of Maastricht (the Netherlands), I would like to thank Nicolaas EP Deutz and his associates for performing sample analysis (HPLC and LCMS) and allowing me to bring back their novel 'reversible Acetaminophen-induced acute liver failure mouse model' to the Institute. I would like to thank Dr Andrew Pitsillides and Robin Soper at the Royal Veterinary College in London who taught me Western-blot techniques that were later successfully introduced to the Institute. I must also thank Hugh Perry and Tracy Newman at the University of Southampton, who continue to collaborate on immunohistochemical studies on our liver failure models.

It is important to state that this thesis would not have been possible without funding from a number or sources - the European Association for the Study of Liver Disease, the Clinical Research and Development Committee at UCL and also industry.

Furthermore, Professor Jalan, Professor Williams (The head of the Institute) and Stephen Hodges must be commended for their approach to developing a rounded group of individuals. Together, they have provided *'synergy'* and a framework for

any subsequent achievements I have the good fortune to see through to fruition. This leads on to the genius that is Dr Nathan Davies, the go-to guy, the '*Ask Jeeves*' of the Institute! He is simply the ultimate enabler. Without Dr Davies, there would be little validity to our academic and research pursuits. I must also acknowledge other distinguished and dear colleagues, collaborators and associates who have supported my efforts – Balasubramaniyan Vairappan, Lisa Cheshire, Dr Yalda Sharifi, Abeba Habtesion, Pamela Lecke, Lorette Noiret and Dr Naina Shah for making the Institute fun yet professional. I would further like to individually thank Professor Kevin Moore (Royal Free Hospital, London), Dr John Xiou, Gabrie Ten Have, Stephen Olde Daminke, Fausto Andreola, Raymond Moss, Celia, Claudia Zwingmann and Heather Brooks for their contribution to this thesis. I must also highlight the role of my close friends who have taken a back seat, but kept smiling - thank you.

Ultimately however, my achievements have only ever been possible due to the unfailing support of my fiancée Tabassan and my family who have guided me through every conceivable obstacle. I would like to highlight how much Tabassan has been instrumental in my career by keeping me motivated and sharing my love of medicine and life. My Mother and Father in particular deserve every possible accolade! I'm grounded because they are! My Sister Karen, Brother Darren and Aunt Marlene have all been very supportive - thank you! Lastly, but most importantly, my Daughter Kaylin who keeps me inspired.

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